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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF
GRASSBERGER ET AL.
APPLICATION NO: 08/471,146
FILED: JUNE 6, 1995
FOR: NEW USE OF 11, 28-DIOXA-4-AZATRICYCLO [22.3.1.04,9] OCTACOS-18-ENE DERIVATIVES AND PHARMACEUTICAL COMPOSITIONS CONTAINING THEM

Art Unit: 1614
Examiner: J. Goldberg

Assistant Commissioner for Patents
Washington, D.C. 20231

APPEAL BRIEF

Sir:

This brief is submitted in support of Appellants' appeal filed September 24, 2002 from the final rejection of claims 29-48 as set forth in the Advisory Action dated September 13, 2002.

REAL PARTY IN INTEREST

The real party in interest in the instant Appeal is Novartis AG, a company organized under the laws of the Swiss Confederation, of Basle, Switzerland.

RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences known to Appellants, Appellants' legal representatives, or assignee which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

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STATUS OF THE CLAIMS

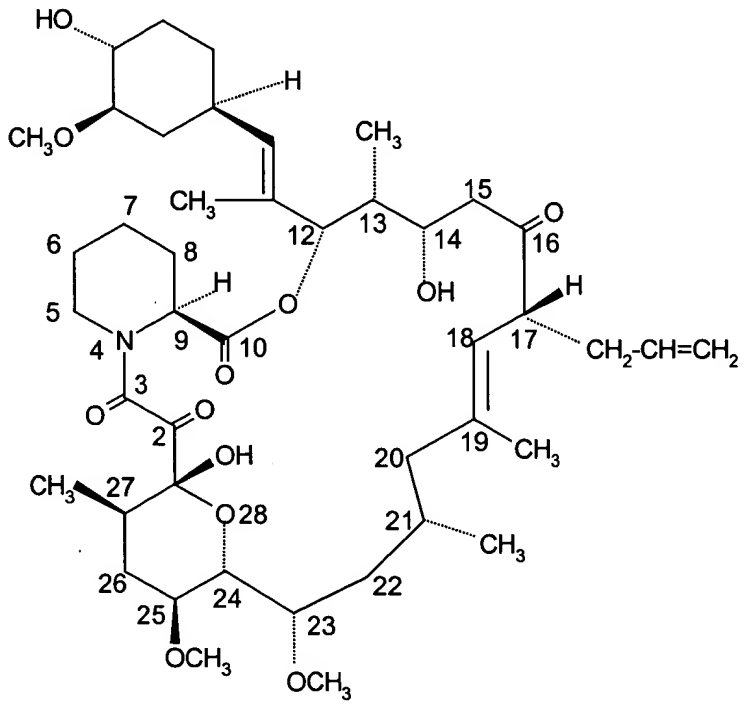
Claims 29-48 are pending in this application and stand finally rejected on art grounds under 35 U.S.C § 103. As noted in the Final Rejection of May 24, 2002, the claims numbered 27-46 in the Amendment of April 26, 2002 have been renumbered as 29-48.

STATUS OF THE AMENDMENTS

No amendments to the claims after Final Rejection have been filed.

SUMMARY OF THE INVENTION

The presently claimed invention is directed to pharmaceutical compositions for topical administration in the form of a lotion, gel, or cream comprising 1% to 3% of a compound of the formula



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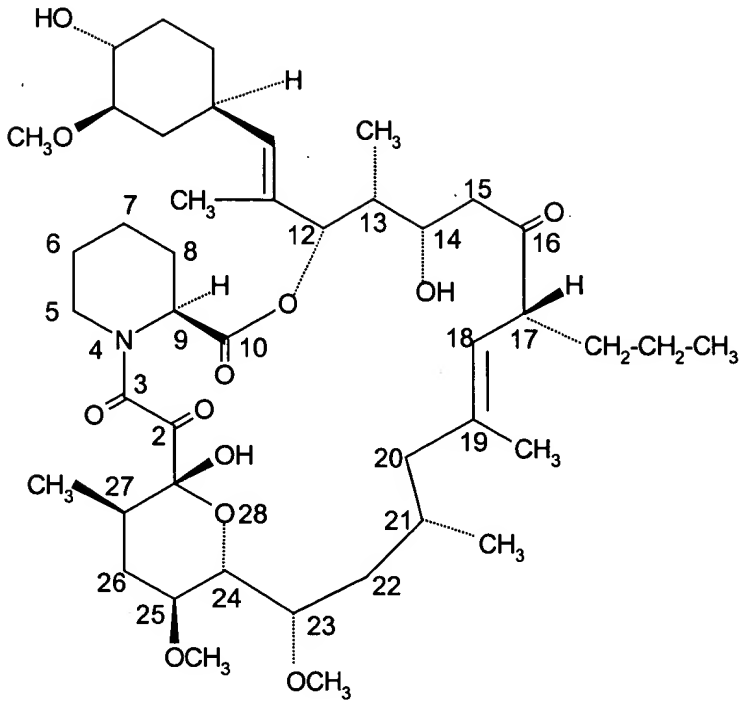
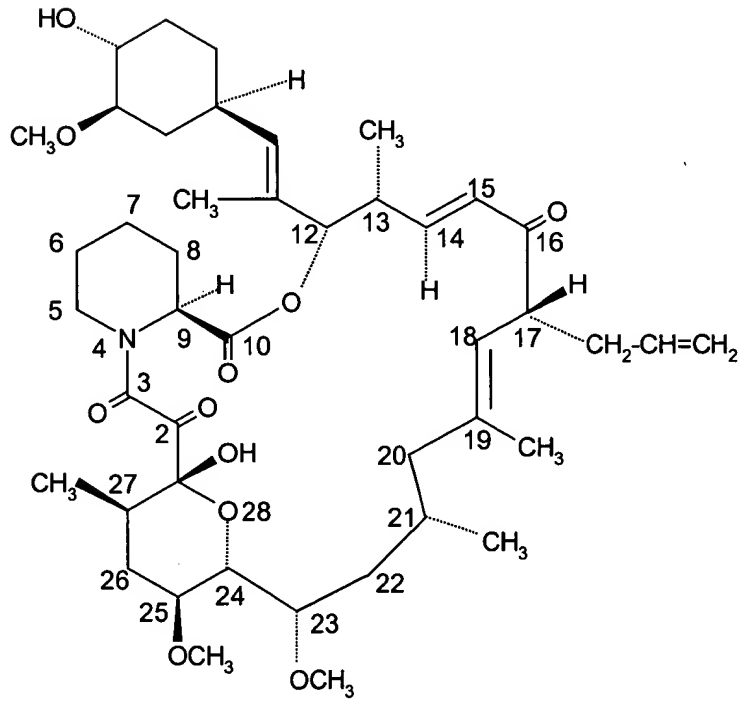
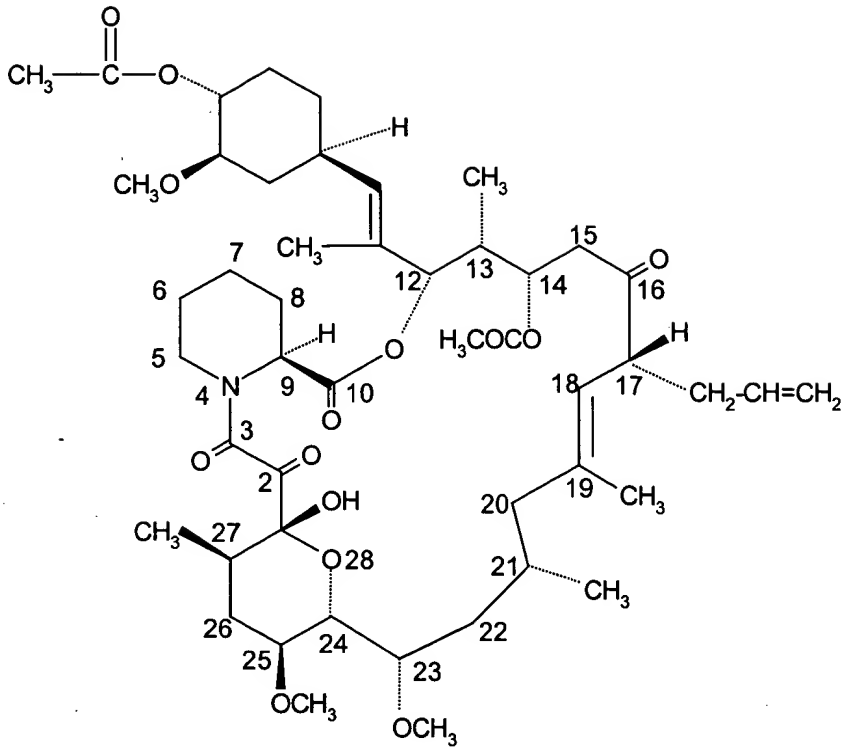
or of the formula



or of the formula



or of the formula



and a pharmaceutically acceptable carrier for a lotion, gel or cream said carrier being a carrier for topical administration.

ISSUES

The instant appeal presents for resolution the following issue involving patentability and the applicability of the provisions of 35 U.S.C. §103(a). Are claims 29-48 properly rejected under 35 U.S.C. §103(a) as being unpatentable over EPA 0,184,162 in view of Johnson (U.S.P.N. 4,411,893) and Showalter (U.S.P.N. 4,556,654). Specifically, the relevant issue is whether the instant composition is prima facie obvious over EPA '162 in view of Johnson and Showalter. The Examiner argues that EPA '162 describes appellants' compounds and that the prior art (of Johnson and Showalter) "is well aware of how antimicrobial agents are applied topically using lotion, gels and creams".

GROUPING OF THE CLAIMS

Claims 29-48 stand or fall together.

ARGUMENT

1. THE REJECTION OF CLAIMS 29-48 OVER EPA 0,184,162 IN VIEW OF JOHNSON (U.S.P.N. 4,411,893) AND SHOWALTER (U.S.P.N. 4,556.654) IS IMPROPER

The rejection of claims 29-48 under 35 U.S.C. 103 (a) as being unpatentable over the teachings of EPA 0,184,162 in view of Johnson and Showalter is respectfully traversed. It is the Examiner's position that EPA '162 describes appellants' compounds, as well as the use of those compounds, "as an antimicrobial agent to be applied with a "cancer" (sic) (e.g. carrier) externally i.e. topically." It is further submitted that the "prior art is well aware of how antimicrobial agents are applied topically using lotion, gels and creams" as shown by Johnson and Showalter.

Appellants' respectfully disagree with the Examiner's interpretation of EPA '162 "external application" as being synonymous/interchangeable with the instant "topical administration." The present claims are directed to **topical** compositions of specific compounds. The topical administration of these compositions produces a local effect (i.e. an effect at the site of administration on the skin) and as such, are used to treat "local conditions", e.g. psoriasis, dermatitis, urticaria, etc. More specifically, the topical compositions are for "the topical treatment of inflammatory and hyperproliferative skin diseases and of cutaneous manifestations of immunologically-mediated illnesses." See page 2, lines 8-10.

In contrast, while EPA '162 does disclose the compounds of the instant invention, there is no disclosure of a **topical** composition as described above. It is not the intention of EPA '162 to topically administer compounds to yield a local effect (e.g. an effect on the skin). EPA '162 does not describe, disclose or suggest "skin diseases" or "cutaneous manifestations". The EPA external compositions are used to treat "systemic" conditions at a distant site, which is to say the compositions yield a systemic effect, e.g. prevention of resistance by transplantation, treatment of graft versus host disease, auto-immune diseases etc. (see page 66, line 33 through page 67, line 6). It is difficult to imagine that these conditions would be effectively treated by a topical composition which would produce a local effect. Those conditions call for systemic administration of active agents, which may be accomplished via an "external" route/composition such as intranasal, buccal, rectal, etc., not by a topical composition. For example, the indication "**systemic** Lupus erythematosus" (page 67, line 3) is an inflammatory autoimmune disease that has, as its name indicates, predominantly systemic implications (see e.g. The Merck Manual of Diagnosis and Therapy [1992] 16th edition, p. 1317-1320). This disease is therefore treated systemically (see pages 1319 and 1320). Copy enclosed for the convenience of the Examiner in the Response After Final Rejection mailed August 26, 2002.

Appellants acknowledge that EPA '162 discloses tricyclo compounds having antimicrobial activity. Appellants further acknowledge that topical compositions of antimicrobial compounds are known. It is Appellants position however, that antimicrobial agents may be formulated for either systemic or topical application, and that nothing in EPA '162 directs one of ordinary skill in the art to formulate the tricyclo compounds into topical compositions. Attention is particularly directed to EPA '162, page 70, Test 2. The fungi used to test antimicrobial activity of the compounds are *Aspergillus fumigatus* and *Fusarium oxysporum*. These organisms are typically treated systemically, not topically. Support for this statement may be found in articles such as "Use of nebulised liposomal amphotericin B in the treatment of A*spergillus fumigatus* empyema" (Thorax, 1995: 50: 1321-1323) and "MENACING MOLD: The Molecular Biology of *Aspergillus fumigatus*" which describes that the fungi causes "systemic infections with high mortality rates." See page 2, first full paragraph. Additionally, as set forth in "Biological Weapons—A Primer for Microbiologists", the trichothecene mycotoxin from *Fusarium* is a potential biological weapon. See page 2, second full paragraph. Copies enclosed for convenience. Thus, while it is well known that antimicrobial compounds may be applied to the skin, one of ordinary skill in the art would not contemplate a topical composition of the tricyclo compounds for the treatment of the potent fungi studied in Test 2 of EPA '162. Additionally, it is observed that no testing was performed in EPA '162 with organisms that would show topical activity such as dermatophytes or yeast fungus.

It is further noted that all *in vivo* tests in EPA'162 refer to systemic application (page 71, line 15 to page 75, line 36). Additionally, the specification, on page 76, lines 32-34, states that "[F]or applying this composition to humans, it is preferable to apply it by parenteral or enteral administration."

Finally, it is noted that nowhere in EPA '621 is a lotion, gel or cream composition taught or suggested. Johnson and Showalter are relied upon as secondary references to show that "the prior art is well aware of how antimicrobial compounds are administered topically in lotions, gels and creams." It is Appellants' position, however, that while the instant compounds are disclosed in EPA '621 and lotions, gels, or creams are disclosed in Johnson and Showalter, there is no motivation to combine these references. EPA '621 does not suggest topical compositions of lotions, gels, or creams nor would EPA '621 desire compositions of lotions, gels, or creams to treat systemic lupus, autoimmune disease, etc. Similarly, neither Johnson nor Showalter suggest the desirability of a topical lotion, gel or cream composition for compounds which are useful for the treatment of systemic conditions.

With regard to Makino (U.S.P.N. 4,871,723) cited by the Examiner in the Advisory Action of September 13, 2002, the Examiner has taken the position that Makino teaches that the term "external" can be applied to the skin. However, it is Appellants' position that such a deliberate definition (i.e. "externally applying to the skin"—Abstract, lines 1 and 2) is evidence that "externally applying" clearly contemplates applications other than to the skin.

CONCLUSION

For the foregoing reasons, claims 29-48 are respectfully submitted as defining subject matter patentably distinguishable from EPA 0,184,162 in view of Johnson and Showalter. Accordingly, it is respectfully requested that the Honorable Board reverse the grounds of rejection set forth by the Examiner so that claims 29-48 may be allowed to proceed to issuance.

In accordance with 37 C.F.R. §1.192(a), two additional copies of this Appeal Brief are being submitted herewith.

Please charge $320.00, the fee required by 37 C.F.R. §1.17(a) upon the filing of an Appeal Brief, to Deposit Account No. 19-0134 in the name of Novartis Corporation. Two copies of this page are appended for purposes of charging such fee. If any additional fees are due, authorization is hereby given to charge such fees to Deposit Account No. 19-0134 in the name of Novartis Corporation.

Respectfully submitted,

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Patent and Trademark Dept.
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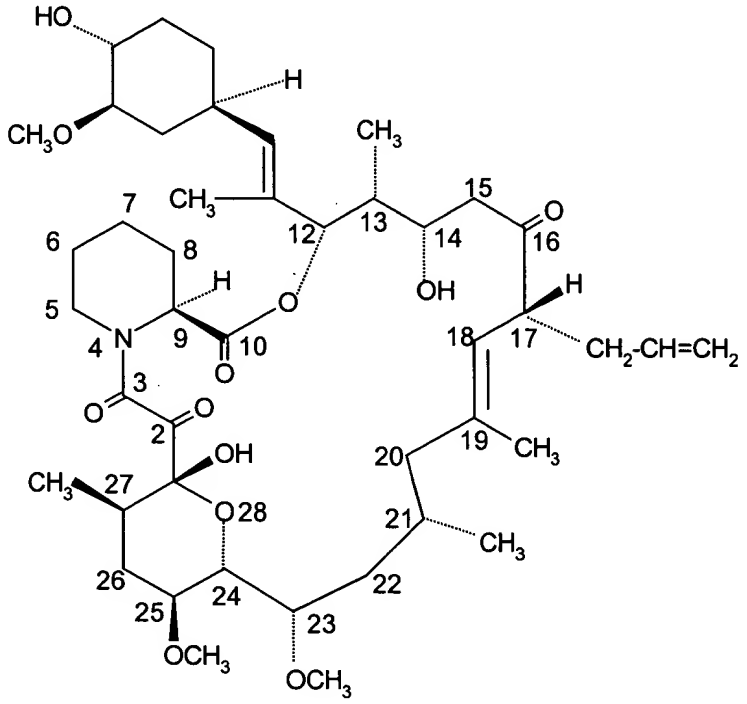
D. Gabrielle Brouillette
Agent for Applicants
Reg. No. 51,384

Date: November 25, 2002

Enclosures: Copies of Brief (3)
Page 8 of Brief in duplicate
Appendix of the claims
Three (3) Journal articles

APPENDIX OF THE CLAIMS

-- 29. A pharmaceutical composition for topical administration in the form of a lotion, gel or cream comprising 1-3% of a compound of the formula



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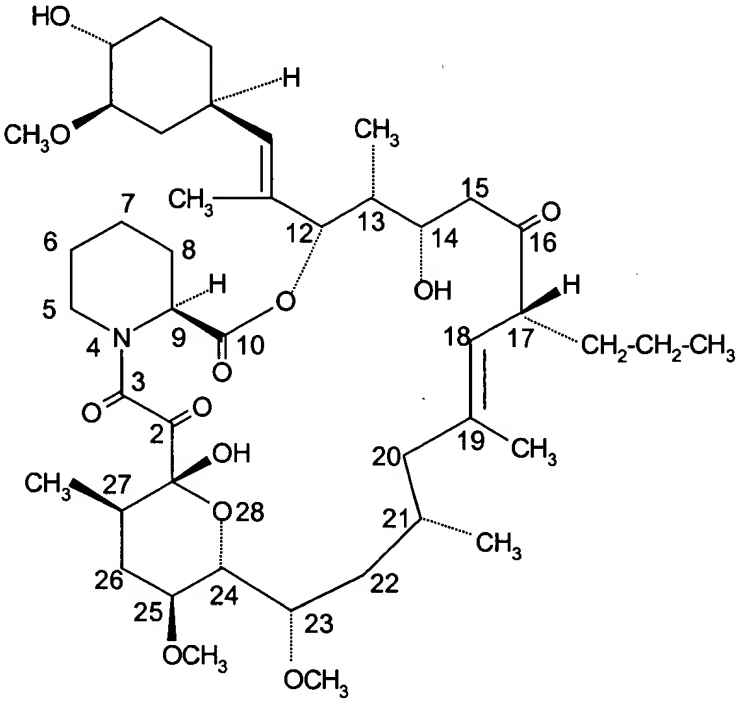
and a pharmaceutically acceptable carrier for a lotion, gel or cream, said carrier being a carrier for topical administration. --

--30. A pharmaceutical composition for topical administration in the form of a lotion, gel or cream comprising 1-3% of a compound of the formula



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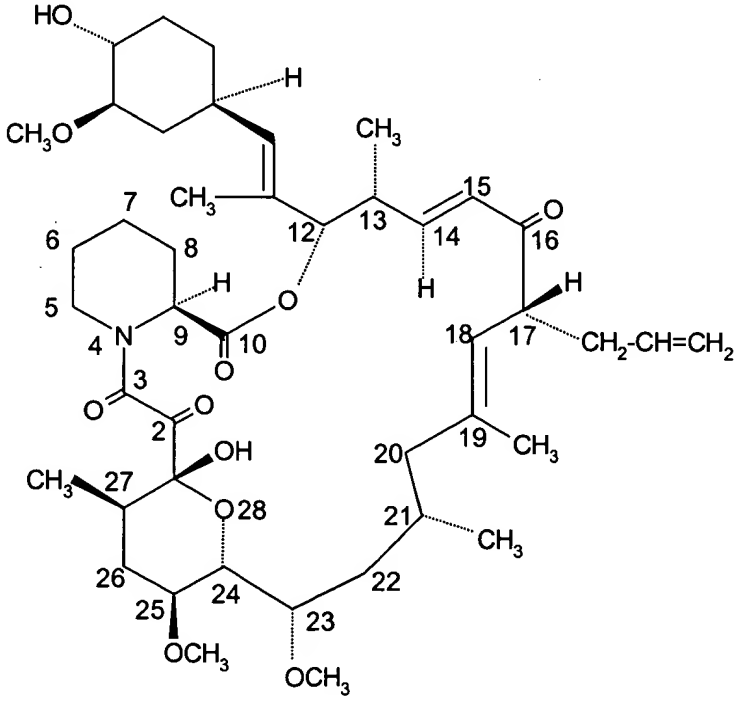
-- 31. A pharmaceutical composition for topical administration in the form of a lotion, gel or cream comprising 1-3% of a compound of the formula



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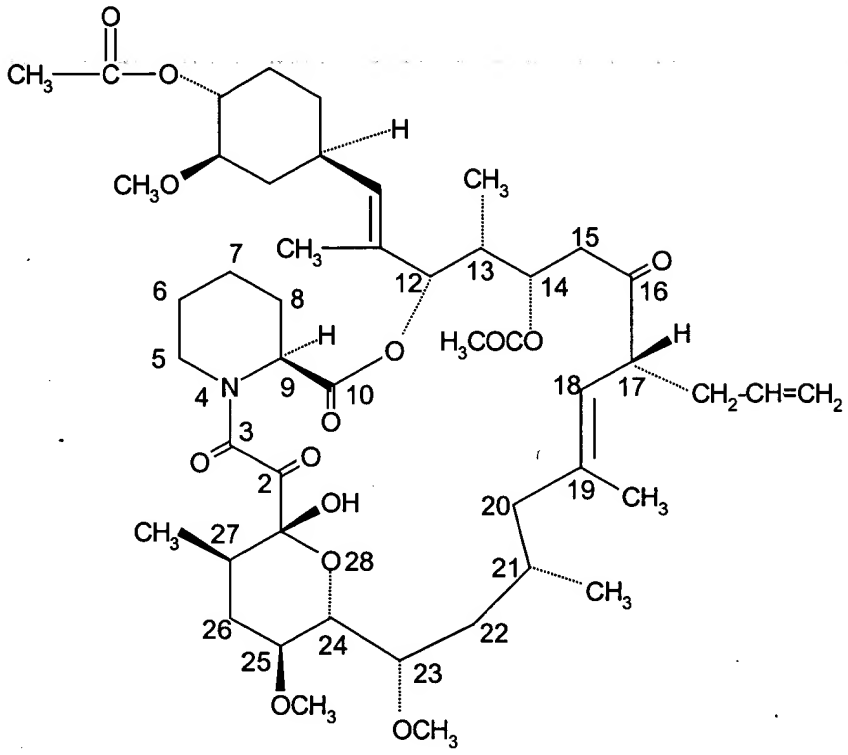
-- 32. A pharmaceutical composition for topical administration in the form of a lotion, gel or cream comprising 1-3% of a compound of the formula



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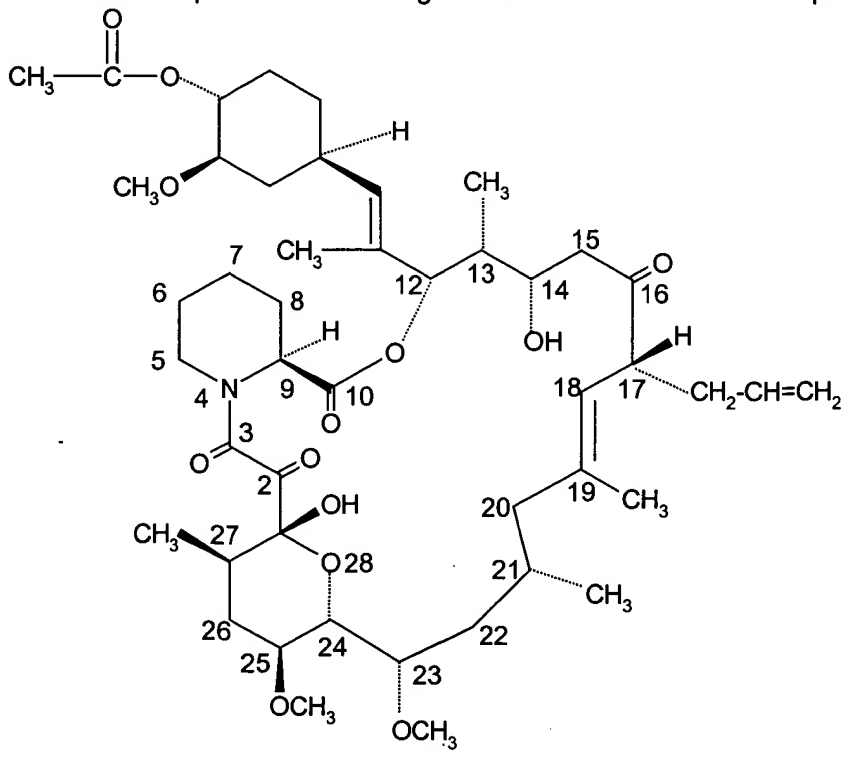
-- 33. The pharmaceutical composition according to claim 19 in which the compound is



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-- 34. A pharmaceutical composition according to claim 27 in which the composition is a lotion. --

-- 35. 5A pharmaceutical composition according to claim 27 in which the composition is a gel. --

-- 36. A pharmaceutical composition according to claim 27 in which the composition is a cream. --

-- 37. A pharmaceutical composition according to claim 28 in which the composition is a lotion. --

-- 38. A pharmaceutical composition according to claim 28 in which the composition is a gel. --

-- 39. A pharmaceutical composition according to claim 28 in which the composition is a cream. --

-- 40. A pharmaceutical composition according to claim 29 in which the composition is a lotion. --

-- 41. A pharmaceutical composition according to claim 29 in which the composition is a gel. --

-- 42. A pharmaceutical composition according to claim 29 in which the composition is a cream. --

-- 43. A pharmaceutical composition according to claim 30 in which the composition is a lotion. --

-- 44. A pharmaceutical composition according to claim 30 in which the composition is a gel. --

-- 45. A pharmaceutical composition according to claim 30 in which the composition is a cream. --

-- 46. A pharmaceutical composition according to claim 31 in which the composition is a lotion. --

-- 47. A pharmaceutical composition according to claim 31 in which the composition is a gel. --

-- 48. A pharmaceutical composition according to claim 31 in which the composition is a cream. --

This report suggests that directly instilled rhDNase may be one alternative to conventional therapy for lobar atelectasis. Its use may be beneficial for the subset of patients with lobar atelectasis due to retained secretions which fail to clear with vigorous coughing, chest physiotherapy, and suctioning. Direct bronchoscopic instillation offers the advantage of concentrating it at the site of obstruction and avoids the delay of waiting for the effects of nebulised administration. Further study is warranted comparing rhDNase by direct instillation and nebulisation with traditional respiratory therapy for lobar collapse due to retained secretions.

1 Armstrong JB, White JC. Liquefication of viscous purulent exudate by deoxyribonuclease. *Lancet* 1950;ii:739–42.
2 Potter JL, Spector S, Matthews LW, Lemm J. Studies on pulmonary secretions: III The nucleic acids in whole pulmonary secretions from patients with cystic fibrosis, bronchiectasis, and laryngectomy. *Am Rev Respir Dis* 1969;9: 909–16.
3 Shak S, Capon DJ, Hellniss R, Marsters SA, Baker CL. Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proc Nat Acad Sci USA* 1990;87: 9188–92.
4 Fuchs HJ, Borowitz D, Christiansen D, Morris E, Nash M, Ramsey B, *et al.* Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. *N Engl J Med* 1994;331:637–42.
5 Hubbard RC, McElvaney NG, Birrer P, Shak S, Robinson WW, Margaret CJ, *et al.* A preliminary study of aerosolized recombinant human deoxyribonuclease I in the treatment of cystic fibrosis. *N Engl J Med* 1992;326:812-5.
6 Marini JJ, Pierson DJ, Hudson LD. Acute lobar atelectasis: a prospective comparison of fiberoptic bronchoscopy and respiratory therapy. *Am Rev Respir Dis* 1979;119:971–8.
7 Anderson JB, Olesen KP, Eikard B, Jansen E, Qvist J. Periodic continuous positive airway pressure, CPAP, by mask in the treatment of atelectasis. *Eur J Respir Dis* 1980;61:20–5.
8 Ricksten S, Bengtsson A, Soderberg C, Thorden M, Kvist H. Effects of periodic positive airway pressure by mask on postoperative pulmonary function. *Chest* 1986;89:774–81.
9 Shah PL, Scott SF, Hodson ME. Lobar atelectasis in cystic fibrosis and treatment with recombinant human DNase I. *Respir Med* 1994;88:313–5.

Thorax 1995;50:1321–1323

Use of nebulised liposomal amphotericin B in the treatment of *Aspergillus fumigatus* empyema

Ian F Purcell, Paul A Corris

Abstract

A 28 year old man with asthma, bronchopulmonary aspergillosis, pulmonary thromboembolic disease, and pulmonary hypertension developed *Aspergillus fumigatus* empyema complicating a pneumothorax. His condition progressively deteriorated despite treatment with intravenous and intrapleural amphotericin B, but improved promptly after substituting nebulised liposomal amphotericin B and oral itraconazole. This experience suggests that nebulised liposomal amphotericin B is well tolerated and merits further assessment in the treatment of pulmonary fungal disease.

(*Thorax* 1995;50:1321–1323)

Keywords: *Aspergillus fumigatus*, empyema, liposomal amphotericin B, nebuliser.

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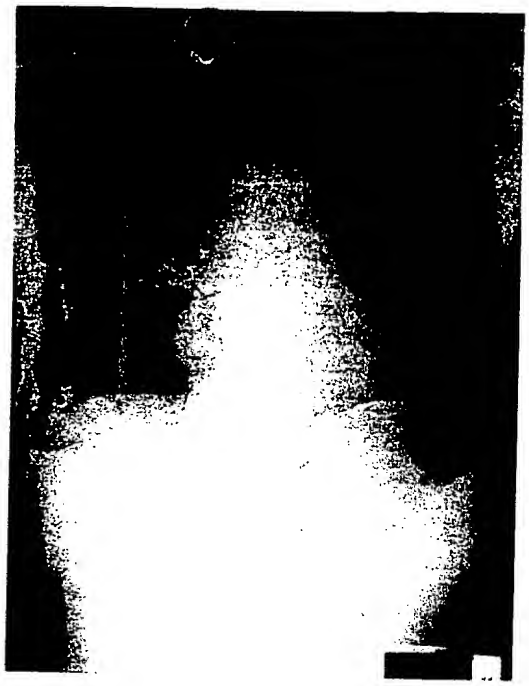
18 April 1995

Aspergillus empyema is a rare condition which predominantly affects patients with chronic lung damage associated with previous tuberculosis and immunocompromised hosts.[1] Liposomal amphotericin B (AmBisome) is an effective agent for the treatment of patients with systemic fungal disease and has been reported as having less toxicity than amphotericin when given intravenously.[2] We report the first clinical use of liposomal amphotericin B delivered directly to the lungs using a nebuliser.

Case report

A 28 year old man had presented seven years previously with increasing dyspnoea. He was known to have asthma which had been well controlled and examination suggested the presence of pulmonary hypertension. Investigation including echocardiography, radionucleotide imaging, and cardiac catheterisation was performed which confirmed the presence of moderately severe pulmonary hypertension; no cause was identified. A diagnosis of unexplained or primary pulmonary hypertension was made and he was anticoagulated with warfarin.

There was a slow gradual deterioration in his exercise tolerance to New York Heart Association III over the next seven years until he presented as an emergency with severe haemoptysis. A chest radiograph at this time demonstrated a cavitating lesion in the left mid zone. His *Aspergillus* precipitins were strongly positive and he was referred for further assessment and management. A contrast enhanced thoracic computed tomographic scan revealed extensive central pulmonary artery thrombosis and evidence of a mycetoma within a cavity. Lower limb venography showed the presence of venous thrombosis and the cause of his pulmonary hypertension was diagnosed as chronic subacute massive pulmonary embolism. The source of the haemoptysis was thought to be an aspergilloma within an old cavitating pulmonary infarct. Bronchial angiography with embolisation of the vessels supplying the cavity was performed, followed by right heart catheterisation and infusion of streptokinase and plasminogen directly into the pulmonary artery.[3] There was no change in his pulmonary vascular resistance or pressure and pulmonary endarterectomy and insertion of an



Chest radiograph with intercostal drain in situ before treatment with nebulised liposomal amphotericin B. A right sided empyema can be seen with adjacent consolidation and cavitation. The right ventricle and pulmonary arteries are enlarged.

inferior vena cava filter was carried out. He was established on warfarin and aspirin and discharged back to his referring hospital.

Twelve months later he was readmitted with a right pneumothorax, pyrexia, and purulent sputum. An intercostal drain was inserted which continually drained purulent fluid and air, indicative of a bronchopleural fistula (figure). The sputum and pleural fluid were positive for *Aspergillus fumigatus* on culture. All other microbiological culture and serological tests (including mycobacteria) were negative, and there was no evidence of an immunocompromised state. He was commenced on intravenous amphotericin B, 1 mg/kg/day, and intrapleural amphotericin B, 20 mg twice daily, via the intercostal drain. After two weeks of treatment he had not improved and cultures remained positive for *Aspergillus*; moreover, his serum creatinine level had risen from 79 to 164 μmol/l. This was regarded as treatment failure and the medication was changed to oral itraconazole, 400 mg/day, and nebulised amphotericin B, 50 mg twice a day via a Turboturrett 2 nebuliser with a driving flow of 8 l/min. The amphotericin B had to be discontinued because it precipitated severe bronchospasm despite premedication with salbutamol. Accordingly, liposomal amphotericin B was substituted at a dose of 50 mg twice daily with no bronchospasm. Within three days of starting this treatment the patient became apyrexial and after four weeks all sputum and pleural fluid cultures were negative for *Aspergillus*. The intercostal drain was removed by the fifth week and treatment discontinued after six weeks. There has been no relapse after one year of follow up.

Discussion

This case reports the first successful use of nebulised liposomal amphotericin B in the treatment of *Aspergillus* empyema complicating a bronchopleural fistula. *Aspergillus* empyema is an uncommon condition and is associated with previous tuberculous infection, thoracic surgery, or cytotoxic therapy.[1] The most likely source of the empyema presented here is rupture of a cavitating pulmonary infarct colonised by *Aspergillus fumigatus*. The patient was a known asthmatic and the presence of a peripheral eosinophilia, high serum IgE, and positive skin prick tests to *Aspergillus* supported the diagnosis of bronchopulmonary aspergillosis in addition to the aspergilloma.

Use of conventional therapy with intravenous and intrapleural amphotericin B failed to control the *Aspergillus* infection and, moreover, led to a doubling of the creatinine level. Liposomal amphotericin B has been shown to be effective when given intravenously to immunocompromised patients who have failed to respond to conventional amphotericin B.[4–6] It has also been shown to cause less nephrotoxicity.[3] We chose to administer amphotericin B via the nebulised route because we wished to deliver a high concentration to the lung and felt that the large bronchopleural fistula may lead to direct entry to the pleural space. Indeed, amphotericin activity was detected in the pleural fluid by an imidazole resistant *Candida glabata* bioassay after 3–8 days of treatment, after which time there was insufficient pleural fluid for the assay to be performed.

Our patient could not tolerate nebulised amphotericin B because it induced bronchospasm. He was, however, able to tolerate nebulised liposomal amphotericin B and this is the first recorded use of this product in nebulised form in humans. A liposomal vehicle has, however, been well recognised as an attractive means of delivering drugs to the lungs.[7]

The role of nebulised liposomal amphotericin B in the successful management of this patient cannot be fully evaluated because of the concomitant administration of itraconazole which has been shown to be effective in a variety of *Aspergillus* infections,[8] and we do not suggest that this report proves an efficacy for liposomal amphotericin B as monotherapy, or even as part of combination therapy, in *Aspergillus* empyema. We can report that nebulised liposomal amphotericin B was well tolerated by this asthmatic patient and did not lead to any unwanted side effects throughout the six week course of treatment. In particular, renal function was seen to improve to normal levels and there was no hypokalaemia or rise in alkaline phosphatase levels. There was no detrimental effect on pulmonary function. Unfortunately, at approximately £10 000 for the six week course, liposomal amphotericin B was an expensive alternative to conventional amphotericin therapy which would have cost £300 for six weeks at a dosage of 1 mg/kg/day.

Formal studies of nebulised liposomal amphotericin B should be considered to define its role in the management of *Aspergillus* pulmonary conditions.

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Sydney S
London S
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D M Ged

1 Denning DW, Stevens DA. Antifungal and surgical treatment of invasive aspergillosis: review of 2121 published cases. *Rev Infect Dis* 1990;12:1147–201.
2 Meunier F, Prentice HG, Ringden O. Liposomal amphotericin B (AmBisome): safety data from a phase 2/3 clinical trial. *J Antimicrob Chemother* 1991;28(Suppl B): 83–91.
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Thorax 1995;50:1323

Commentary

D M Geddes

These three cases are at first sight completely different, but there is an important common theme. In each instance an unconventional and expensive treatment was given when all else had failed and the results were gratifyingly successful. This begs the question, common to all case reports, whether they are illustrative and helpful for the management of other patients, or whether they simply represent an amazing coincidence which has been given spurious respectability by being published. The clinical teams should be congratulated on their ingenuity and persistence, but did the management change actually contribute to the improvement or was it simply a way of structuring time (at considerable expense) until nature did the job?

The case presented by Codispoti *et al* (pp 1317–9) describes the use of extracorporeal membrane oxygenation (ECMO) to support a patient dying of pneumococcal pneumonia until treatment was effective. The key question here is whether or not she could have survived the six days of ECMO support with conventional ventilation. The reason given for starting ECMO was that the haemodynamic and ventilatory parameters were considered incompatible with survival. If we assume this judgement to be correct, then ECMO undoubtedly saved her life. Previous studies of ECMO in randomised trials have shown no benefit, but the failure of a treatment for a randomised group does not mean that the treatment could never be helpful to anyone. The lesson from this report is that ECMO should be considered as a last ditch means of ventilatory support when there are very good grounds to predict eventual recovery. Such cases are probably very few, and it is essential that this report is not used as an argument for an expensive escalation in preterminal treatment for those patients with no hope of survival.

The second report by Touleimat *et al* (pp 1319–21) suggests that instillation of DNase via a bronchoscope to an area of the lung subject to recurrent collapse from sputum retention is worthwhile. The description of the results "... rapid dissolution of sputum and opening of the orifice ..." is more in line with reporter enthusiasm than enzyme kinetics. Also, at first sight it is difficult to see how a single DNase treatment with clearing of sputum could have a lasting effect when aspiration of the plug previously did not. It is conceivable, however, that DNase allowed clearance of more peripheral airways and thus promoted better ventilation and clearance to prevent subsequent sputum plugging. DNase is expensive for chronic treatment but a single dose is not, and this report certainly suggests a possible indication for a new drug over and above that which has been established in clinical trials. It is interesting to speculate whether nebulised DNase would have been equally successful, avoiding repeat bronchoscopies with the cost and inconvenience involved.

The final case report described by Purcell and Corris on pp 1321–3 is the most difficult to believe. In this, nebulised liposomal amphotericin apparently sterilised the pleural space when intrapleural instillation of amphotericin had failed. Even if the patient did have a large bronchopleural fistula, this still sounds a singularly inefficient way of getting a drug into this compartment. Either there is some additional therapeutic benefit from putting the drug into a liposome, which is highly unlikely, or the itraconazole that was given at the same time was actually responsible for the improvement. Either way the main interest of the report, as the authors point out, is that the liposomal form of amphotericin did not provoke asthma when the conventional form had done so, and there may well be other situations in which liposomal amphotericin by nebuliser would be worth considering.

Taken together, these reports are useful for clinicians to remember when they have a very difficult case on their hands. It is, however, essential that these approaches are applied intelligently and are not used as an excuse for mindless expense. Controlled clinical trials are valuable for common problems but cannot address rare and difficult management issues such as these. This is the true value of case reports.

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MENACING MOLD: The Molecular Biology of *Aspergillus fumigatus*

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KEY WORDS: ***Aspergillus fumigatus***, molecular techniques, genomics, virulence

ABSTRACT

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Infections with mold pathogens have emerged as an increasing risk faced by patients under sustained immunosuppression. Species of the ***Aspergillus*** family account for most of these infections, and in particular ***Aspergillus fumigatus*** may be regarded as the most important airborne pathogenic fungus. The improvement in transplant medicine and the therapy of hematological malignancies is often complicated by the threat of invasive aspergillosis. Specific diagnostic methods are still limited as are the possibilities of therapeutic intervention, leading to the disappointing fact that invasive aspergillosis is still associated with a high mortality rate that ranges from 30% to 90%. In recent years considerable progress has been made in understanding the genetics of *A. **fumigatus***, and molecular techniques for the manipulation of the fungus have been developed. Molecular genetics offers not only approaches for the detailed characterization of gene products that appear to be key components of the infection process but also selection strategies that combine classical genetics and molecular biology to identify virulence determinants of *A. **fumigatus***. Moreover, these methods have a major impact on the development of novel strategies leading to the identification of antimycotic drugs. This review summarizes the current knowledge on the biology, molecular genetics, and genomics of *A. **fumigatus***.

INTRODUCTION

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In the past 20 years the deuteromycete ***Aspergillus fumigatus*** has gone from being a saprophytic fungus of minor interest to scientists to becoming one of the most important fungal pathogens. The main reason for the rise in systemic infections lies in the steady increase in the number of immunocompromised individuals, the main risk group for such infections (51). Diseases caused by *A. **fumigatus*** can be divided into three categories: (*a*) allergic reactions and (*b*) colonization with restricted invasiveness are observed in immunocompetent individuals, whereas (*c*) systemic infections with high mortality rates occur in immunocompromised patients. Added to this is the lack of effective therapy, resulting in a high mortality rate between 30 and 90% (32).

Although *A. **fumigatus*** only makes up a small proportion of all aerial spores, around 0.3% in the air of a particular hospital, it causes roughly 90% of systemic ***Aspergillus*** infections (73). This suggests that *A. **fumigatus*** possesses certain factors that allow it to become an opportunistic human pathogen in immunocompromised patients. In recent years great progress has been made in understanding the genetics of *A. **fumigatus***; molecular genetic techniques have been developed that allow a detailed characterization of the fungus. Molecular biology provides effective techniques for understanding the mechanisms underlying *A. **fumigatus*** virulence and for investigating the key components of the infectious process.

In this review we present an overview of the molecular biological technology available for studying *A. **fumigatus*** and for identifying possible virulence determinants. Most importantly, gene transfer methods for *A. **fumigatus*** are available, some of which were first developed for other ***Aspergillus*** species such as *A. nidulans* and *A. niger* (55). Unlike its relative *A. nidulans*, *A. **fumigatus*** has not revealed a sexual reproduction cycle; however, some classical genetic studies are possible. In addition, a large range of molecular biological tools is now available for investigating *A. **fumigatus***. The most important of these tools are described here. In conjunction with the genome sequence of *A. **fumigatus***, these should eventually lead to the discovery of the key elements of *A. **fumigatus*** virulence and eventually to drugs for treating systemic *A. **fumigatus*** infections. Further general information can be obtained from the ***Aspergillus*** web site (http://www.aspergillus.man.ac.uk/). A comprehensive overview about putative virulence determinants of *A. **fumigatus*** has been given in several reviews (10, 11, 45, 51, 52).

BIOLOGY AND GENETICS OF *ASPERGILLUS FUMIGATUS*

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The natural ecological niche of the saprobic fungus *A. **fumigatus*** is the soil. By contributing to the degradation of decaying organic matter such as compost and hay, *A. **fumigatus*** plays an important role in recycling carbon and nitrogen sources (24, 58, 82).

*A. **fumigatus*** conidia (spores) are gray-green in color and only 2.5–3.0 μm in diameter, allowing them to reach the lung alveoli (65, 69, 70) (Figure 1). Conidia contain a single haploid nucleus. The conidiophore extends from a foot cell at right angles to the mycelium and culminates in a broadly clavate vesicle (20–30 μm in diameter). A single layer of cells (phialides) is attached to

the apical side of the vesicle. The conidia result from a constriction of an elongated portion of the phialide (13) (Figure 2).

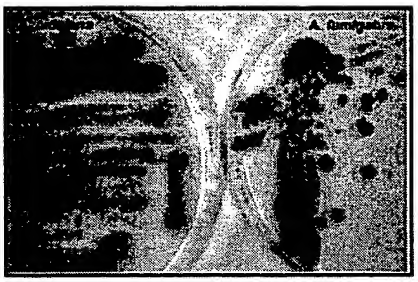

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Figure 1. Sporulating colonies of ***Aspergillus fumigatus*** (*right*) and ***Aspergillus nidulans*** (*left*) growing on ***Aspergillus*** minimal medium agar plates. The characteristic *gray-green* color of *A. **fumigatus*** and the green color of *A. nidulans* result from conidial pigment.

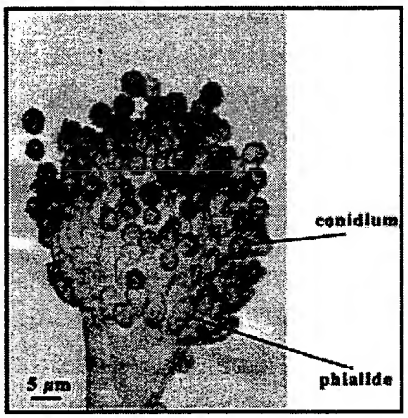

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Figure 2. Scanning electron microscope image of an ***Aspergillus fumigatus*** conidiophore showing the stem cell, phialides, and conidia.

*A. **fumigatus*** is able to grow rapidly on minimal agar plates containing a carbon source (e.g., glucose), a nitrogen source (e.g., nitrate), and trace elements. In addition to being relatively flexible with regard to growth medium, *A. **fumigatus*** is also able to withstand high temperatures. Growth occurs up to 55°C, and conidia are able to survive at temperatures up to 70°C [reviewed in (51, 70)].

The genome of *A. **fumigatus*** has not yet been fully characterized, but its size is estimated at around 27.8 Mb (http://www.tigr.org/), about the same as calculated for *A. nidulans* (14). Pulse-field gel electrophoresis has shown the presence of 5 chromosomal DNA bands ranging in size from 1.7 to 4.8 Mb (80). A first approximation suggests that the genomes of *A. nidulans* and *A. **fumigatus*** are in fact similar (2). A full genome sequencing project is under way for *A. **fumigatus*** (see section Genomics).

CLASSICAL GENETIC TECHNIQUES

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The fact that *A. **fumigatus*** has haploid uninucleate conidia facilitates the isolation of clones and the analysis of mutants by classical or molecular techniques. The existence of a teleomorph form of *A. **fumigatus*** is still under debate. However, DNA-DNA reassociation values less than 70% were found for *A. **fumigatus*** and *Neosartorya*, proving conclusively that *Neosartorya* is not the teleomorph form of *A. **fumigatus*** (64). Other analyses using different methods produced identical results (38). Unlike *A. nidulans*, which has a well-characterized sexual cycle, no sexual cycle has been described for *A. **fumigatus***. Because a sexual cross is necessary to generate linkage data for different genetic loci, the amount of linkage information available for *A. **fumigatus*** has been restricted. However, in the early 1960s parasexuality was described for *A. **fumigatus*** (5, 77). The parasexual capability of *A. **fumigatus***, in which two fungal strains growing in close proximity can be induced to form a heterokaryon (the frontier method), results in diploid nuclei that can be maintained as a stable diploid form. Treatment with a destabilizing agent (e.g., benomyl) results in haploid strains [reviewed in (63)]. Reports by de Lucas et al. (27) and Brookman & Denning (15) suggest that parasexuality in *A. **fumigatus*** can be a powerful technique. It could be used to exchange marker genes between strains. New auxotrophic mutants of *A. **fumigatus*** could help to establish this technique.

MOLECULAR GENETICS

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Transformation Systems

TRANSFORMATION PROCEDURES

Most of the transformation protocols currently used for *A. **fumigatus*** are based on methods developed for transformation of *A. nidulans* and *A. niger* in the early 1980s (4, 18, 34). The most frequently used method is the transformation of protoplasts following cell wall degradation by a lytic enzyme preparation. Both Glucanex (Novo Nordisk) and Panzym (Begerow) have been used successfully. Protoplasts are maintained in an osmotically stabilized solution containing PEG/$CaCl_2$ in order to induce the uptake of DNA into the cells. Subsequently protoplasts are regenerated on osmotically stabilized medium containing the relevant antibiotic or lacking the necessary nutritional supplement, depending on the selection marker being used. Transformation efficiencies are in the range of 1–100 transformants/μg DNA. Although this method is reliable, it does have several drawbacks. The low transformation efficiency can be a problem for complementing mutant alleles, while the method is time consuming. Unfortunately *A. **fumigatus*** protoplasts are not amenable to freezing (K. Langfelder, unpublished observation).

Electroporation of germinating conidia is another method that can be used to transform *A. **fumigatus***. This method had previously been described for other filamentous fungi, including *A. oryzae* (20, 21), *A. niger* (61), and *A. nidulans* (71). Recently Weidner et al. (85) demonstrated that this technique can also be used for transformation of *A. **fumigatus***. In *A. oryzae* the method is significantly quicker than the transformation of protoplasts because conidia do not require treatment with lytic enzymes and transformant colonies appear sooner (61). Pretreatment with cell wall–degrading enzymes increased transformation efficiencies to 100 transformants/μg DNA. In

*A. **fumigatus*** transformation efficiencies are 2- to 10-fold times higher using electroporation than for the protoplast-forming method (31). However, it appears that the percentage of ectopic integrations into the genome is significantly higher when the electroporation method is used. This means that electroporation is not the method of choice when site-directed integration or single-copy integration is required, unless it is possible to select for specific integration sites.

de Groot et al. (25) demonstrated that it is also possible to use *Agrobacterium tumefaciens* to transform filamentous fungi. In ***Aspergillus awamori*** they showed an increase of 600-fold relative to conventional transformation techniques (i.e., protoplast formation and electroporation), with integration in a manner similar to that seen in plants (i.e., a single copy of the Ti-plasmid integrates at random at a chromosomal locus). They also demonstrated that the technique is applicable to filamentous fungi in general by successfully transforming *A. niger*, *Trichoderma reesei*, and *Neurospora crassa*, among others. Despite the utility of such a method few publications report the actual use of the method to transform filamentous fungi (40); not one describes its use in *A. **fumigatus***.

DOMINANT SELECTABLE MARKERS

*A. **fumigatus*** and a number of other filamentous fungi are sensitive to several antibiotics such as phleomycin/bleomycin and hygromycin B. Bacterial genes that produce resistance to such compounds can be used as dominant selectable markers in filamentous fungi. For *A. **fumigatus*** the most frequently used resistance gene is the hygromycin B phosphotransferase (*hph*) gene of *Escherichia coli*. Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus*, which inhibits protein synthesis in prokaryotic and eukaryotic cells (39). The protein encoded by the *hph* gene phosphorylates hygromycin B molecules, which results in complete loss of biological activity (62). In order to express this gene in *A. **fumigatus*** and other filamentous fungi, expression cassettes were designed in which expression of the *hph* gene was placed under the control of fungal promoters. One particular cassette in which the *hph* gene is under the control of the strong, constitutive glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) promoter of *A. nidulans* and the *trpC* terminator region of *A. nidulans* (67) has been used frequently for transforming a variety of different filamentous fungi (68). The *ble* gene, which confers resistance to phleomycin/bleomycin, has been used far less frequently for transformation of *A. **fumigatus***. Bleomycin acts by intercalating in DNA, which leads to degradation of DNA (35). The Ble protein binds to bleomycin with high affinity and thus prevents it from interacting with DNA (35). On such occasions where bleomycin has been used, it was often required to generate a second gene deletion in a strain already carrying the *hph* gene (57, 74).

Avalos et al. (3) described using the *bar* gene of *S. hygroscopicus*, which confers resistance to the herbicide bialaphos, as a selectable marker in *N. crassa*. However, hygromycin B resistance is still the dominant marker of choice for *A. **fumigatus*** (31). Although the availability of other dominant selectable markers would provide useful tools for generating multiple deletions in a single strain, in the absence of such markers other techniques can be used, such as "*ura*-blaster" and auxotrophic markers.

AUXOTROPHIC MARKERS

Auxotrophic markers provide an alternative to using dominant selectable markers for transformation of *A. **fumigatus***. Researchers working on *A. **fumigatus*** do not have access to the large collection of auxotrophic mutants that exist for *A. nidulans*. However, new sequence data from the *A. **fumigatus*** sequencing project should make it possible to delete specific genes (e.g., *argB*) known to be essential for *A. nidulans*. In this way, a collection of auxotrophic strains can be generated that can be complemented with the corresponding *A. **fumigatus***, *A. nidulans*, or *N. crassa* genes. In addition to providing additional selectable markers, transformation of auxotrophic mutants also makes other techniques possible (e.g., site-directed integration).

One example of an auxotrophic marker that is available in *A. **fumigatus*** is the *pyrG* gene, which encodes the orotidine-5'-monophosphate decarboxylase enzyme essential for the synthesis of uracil. *pyrG* mutants, auxotrophic for uracil, have been identified among strains that are not sensitive to 5-fluoro-orotic acid (FOA) (8, 29). *pyrG* homologs have been identified in a number of other species as well, including *A. nidulans* (*pyrG*) (60), *A. niger* (*pyrG*) (87), *Candida albicans* (*ura3*) (53), and *Schizosaccharomyces pombe* (*ura4*) (41). van Hartingsveldt et al. (84) developed *pyrG* as a selectable marker for transformation of *A. niger*. Later, Weidner et al. (85) cloned the *A. **fumigatus** pyrG* gene and established a homologous transformation system for *A. **fumigatus*** using *pyrG*. This means that *A. **fumigatus** pyrG* deletion strains can be utilized for both homologous and heterologous transformations with *A. **fumigatus*** or *A. niger pyrG*. In general, the background in transformations using uracil prototrophy as selection marker is relatively low in *A. **fumigatus*** (S. Gattung, K. Langfelder & A.A. Brakhage, unpublished results).

Recently a further homologous recombination system was developed for *A. **fumigatus*** based on the *sC* gene, which encodes ATP sulfurylase (26). The system had been established previously for *A. niger* (19). Fungal strains lacking the gene are incapable of utilizing sulfate as the sulfur source. Instead, they require reduced sulfur (e.g., methionine) as the sulfur source. In addition, they are no longer sensitive to selenate (which is taken up by the same system as sulfur but then becomes toxic after reduction inside the cell). *A. **fumigatus** sC* mutant strains were isolated by selection for selenate resistance. Such strains could be transformed successfully with a plasmid encoding the intact *sC* gene as the selectable marker gene. Transformants regained the ability to grow on minimal media using sulfate as the sole sulfur source. The authors found that transformation efficiencies were similar to those observed using *pyrG* as the selectable marker gene (26).

A further metabolic gene that has been used successfully as a selectable marker in filamentous fungi, although not yet in *A. **fumigatus***, is the *niaA* gene (23, 72, 81). Its product, nitrate reductase, is required for growth when nitrate is the sole nitrogen source. This marker has the added advantage that strains lacking the *niaA* gene are not sensitive to chlorate, whereas strains carrying a functional *niaA* gene are sensitive. Thus it is possible to select for spontaneous *niaA* deletions, which is an advantage. *niaA* has in fact been developed as a selectable marker for *A. niger* (81), and this system could probably be adapted in order to provide an additional selectable marker in *A. **fumigatus***. The *A. **fumigatus** pabaA* gene, encoding the *para*-aminobenzoic acid synthase enzyme, has been cloned (16) and could provide a useful selectable marker. This and a number of other metabolic genes could also be suitable as selectable markers for *A. **fumigatus***. Among them is the *A. nidulans argB* gene, which has been used as a heterologous marker for

transformation of *A. niger* (18) and other genes involved in essential processes.

One possible disadvantage in using auxotrophic markers is complications in testing virulence of deletion mutants. Often auxotrophic strains show a significantly reduced virulence. For example, a strain auxotrophic for uracil (i.e., *pyrG* deletion strain) shows a marked growth defect and was shown to be apathogenic in a murine infection model (29). Likewise, Tang et al. (79) showed that *A. nidulans pabaA* deletion strains are completely apathogenic in a murine infection model. However, they also showed that by supplying the mice with *para*-aminobenzoic acid in their drinking water it was possible to fully restore virulence of the *pabaA* deletion strain. This knowledge could also be applied in order to make "safe" hypervirulent strains of *A. **fumigatus***. Using a *pabaA* deletion strain and supplementing growth media and/or drinking water of test animals with *para*-aminobenzoic acid, one can carry out risk-free experiments with potentially hypervirulent *A. **fumigatus*** strains.

In summary, a number of selectable markers, both dominant and auxotrophic, are available for the transformation of *A. **fumigatus***. As a result *A. **fumigatus*** is accessible to a variety of molecular biology techniques in order to define and investigate potential virulence factors. Even so, it would be advantageous to have a collection of auxotrophic mutants of a single wild-type strain in order to make better use of auxotrophic markers.

COUNTER-SELECTION SYSTEMS

Although in theory there are a number of selection markers available for transformation of *A. **fumigatus***, in practice it is difficult to generate strains with multiple gene deletions. To date only two such strains have been published (57, 74). In both cases hygromycin B and phleomycin were used as selectable markers. But what if three transformations are required?

One solution is to make use of a *ura*-blaster to transform a uracil-auxotrophic strain that has a nonfunctional *pyrG* gene (Figure 3). A *ura*-blaster cassette is similar to a conventional deletion vector in that it contains a selectable marker, in this case the *pyrG* gene, flanked by upstream and downstream sequences of the gene of interest. In addition, the blaster cassette must contain direct repeats in front of and behind the *pyrG* gene to allow recombination to occur. A functional *pyrG*-blaster for *A. **fumigatus*** was established using the *A. niger pyrG* gene as selectable marker and two copies of the gene-encoding neomycin phosphotransferase (*neo*) as direct repeats (28). Following transformation with the *pyrG*-blaster, transformants are selected that are uracil prototroph. These are analyzed to find strains carrying a deletion of the gene of interest. Such strains are plated on medium containing 5-FOA and supplemented with uracil. Only strains that have lost the *pyrG* gene through recombination between the direct repeats will grow under these conditions because a functional orotidine-5'-monophosphate decarboxylase enzyme converts 5-FOA to a toxic product. Such strains can be used for a second round of transformation to delete further genes or redundant genes that might otherwise mask the effect of a single gene deletion (Figure 3). Under these conditions recombination events occur at a frequency as high as 10^{-4} in *A. **fumigatus*** and *A. nidulans* (28, 30).

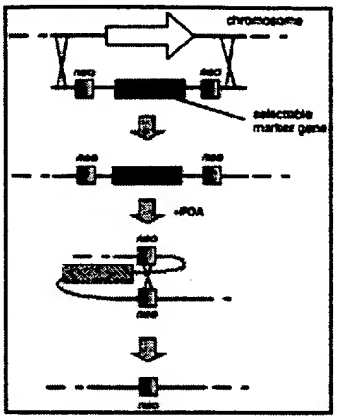

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Figure 3. Schematic representation of the gene deletion method using a *ura*-blaster. The *arrow* marks a region of interest on a fungal chromosome. The *hatched box* represents the selectable marker gene *pyrG*, and the *small shaded boxes* represent the *Escherichia coli* neomycin phosphotransferase gene (*neo*). Following a double homologous recombination, the region of interest is replaced by the selectable marker gene and the flanking *neo* genes. After a transformant has been selected, which carries the required deletion, a negative selection is carried out. This strain is grown in a medium that contains 5'-fluoro-orotic acid (FOA) in addition to uridine. This allows selection of strains that have lost the *pyrG* gene through homologous recombination between the two *neo* gene copies flanking the *pyrG* gene. In such strains the *pyrG* gene can be used as selectable marker gene for further rounds of transformation.

An additional counter-selection system that allows multiple rounds of transformation using the same selectable marker gene is the *sC* system developed in *A. **fumigatus*** by de Lucas et al. (26). As described previously, the lack of a functional *sC* gene (encoding ATP sulfurylase) results in strains that are unable to utilize sulfate as a source of sulfur and therefore require a reduced sulfur source such as methionine. They are also not sensitive to selenate. These properties allow the *sC* selection system to be used in the same way as the *ura*-blaster in order to carry out repeated selection rounds with the *sC* gene as selectable marker. All that would be required to establish the system is a blaster construct analogous to the *ura*-blaster (i.e., with the *sC* gene flanked by two direct repeats, such as the *neo* gene). With this system, *sC* mutants could be transformed using the *sC* gene as the selectable marker gene. Then, transformants could be plated on selenate to select for strains that had lost the *sC* gene again through homologous recombination. These strains can be used in subsequent transformation rounds using the *sC* gene as the selectable marker gene. This should be another useful tool to assist in the functional analysis of the *A. **fumigatus*** genome.

MODE OF INTEGRATION OF DNA

In order to produce genetically stable *A. **fumigatus*** transformants, DNA must be integrated at a chromosomal locus. If the recombinant DNA does not contain homologous sequences, then it can integrate at random in the genome (i.e., ectopically). Although no extensive studies have been carried out, initial experiments in *A. nidulans* suggest that some loci are more favored for homologous recombination than others (7). If the recombinant DNA carries homologous sequences, then integration can occur by homologous recombination. Even if the transforming DNA contains homologous sequences, it still integrates ectopically at high frequency. Bird & Bradshaw (7) showed that in *A. nidulans* the efficiency of homologous recombination correlates with the size of the homologous fragment. In addition, strategies exist that can increase the targeting efficiency at defined genetic loci to almost 50% (85).

AUTONOMOUSLY REPLICATING PLASMIDS

Under normal circumstances stable transformation of *A. **fumigatus*** results from a recombination event taking place between the transforming DNA and one of the fungal chromosomes. The

transforming DNA becomes integrated in the genome. This is useful because it allows the construction of genetically stable transformants. However, this is one of the reasons why the transformation efficiency is relatively low for *A. **fumigatus***.

No replicating plasmids have been isolated from any ***Aspergillus*** species to date. However, Gems et al. (37) detected a sequence of chromosomal origin, AMA1, that allows plasmids carrying this sequence to replicate in *A. nidulans*. The AMA1 sequence is 6.1 kb in length and contains an inverted repeat separated by a unique sequence. The presence of AMA1 on a vector leads to a significant increase in the transformation efficiency (around 250-fold increase over normal transformation efficiencies). AMA1 was also functional in other filamentous fungi including *A. oryzae*, *A. niger*, and *Penicillium chrysogenum* (33). In *A. **fumigatus*** cotransformation of cosmids and a plasmid containing the AMA1 sequence increased transformation efficiency 10-fold (49). de Lucas et al. (26) also reported a 10-fold increase in transformation efficiency when a cotransformation with the plasmid pHELP, which carries the AMA1 sequence, was carried out. This effect can probably be explained by recombination between plasmid and cosmid. Unfortunately cotransformation with plasmids containing the AMA1 sequence frequently leads to rearrangements on the cotransformed cosmid (J. Van den Brulle & A.A. Brakhage, unpublished results). This prevents an otherwise useful tool being employed more often. Nevertheless plasmids containing the AMA1 sequence can be used to clone genes from gene libraries (36) and for other applications (1). Solving the side effects of the AMA1 sequence (genetic instability, rearrangements) would provide an additional tool for investigating *A. **fumigatus***.

Complementation of Mutants

Several techniques exist to create a large number of *A. **fumigatus*** mutants, e.g., restriction enzyme-mediated integration (REMI) (17) or UV-mutagenesis (46). Complementation of mutant phenotypes with libraries of *A. **fumigatus*** or other filamentous fungi can be used to characterize mutations. For example, an *A. **fumigatus*** mutant with white spores could be complemented with the *A. nidulans wA* gene (9). More recently a mutant with an identical phenotype was generated by UV-mutagenesis (46) and complemented with an *A. **fumigatus*** wild-type cosmid library (49). The complementing gene, *pksP*, was indeed found to be the homolog of the *A. nidulans wA* gene, both of which encode a polyketide synthase involved in conidial pigment biosynthesis (56). This example shows that complementation of mutants is a powerful tool for analyzing the functions of specific *A. **fumigatus*** genes given that mutants display a recognizable phenotype.

Other methods for generating a large number of mutants include REMI and transposon mutagenesis. REMI was established in *A. **fumigatus*** by Brown et al. (17) as a method to generate a large population of roughly random mutants. Integrations were distributed throughout the genome, but clustering was observed to some extent. By including the restriction enzymes *Kpn*I or *Xho*I in the transformation, they obtained a large frequency of single-copy integrations in the genome. Transposons have been described for a number of fungi (47). Transposon tagging has been demonstrated successfully in filamentous fungi (48); recently a transposon-mediated mutagenesis was reported in *A. **fumigatus*** (6). This can be a powerful technique for generating a large library of mutant strains, with the added advantage that the site of integration is labeled and can easily be identified.

Knock-Out Versus Gene Disruption

Among the most important methods for determining the function of *A. **fumigatus*** genes and their possible involvement in virulence is the creation of strains in which the relevant gene is no longer functional. In this manner one can study the role of the gene based on the phenotype that results when it is not functional. A second method would be the overexpression of the relevant gene, but this is not always conclusive. Three different approaches can be used to generate strains in which the relevant gene is not functional.

The simplest method is called gene disruption (Figure 4*A*). Here an internal fragment of the gene of interest, often lying toward the 5'-region of the gene, is cloned into a vector carrying a selectable marker. The resulting plasmid is used to transform *A. **fumigatus***. Homologous recombination with the gene of interest via a single crossover results in two mutant copies of the gene separated by the selectable marker. Neither of the genes should encode functional proteins, since both copies are truncated (one lacks the 3' region while the other lacks the promoter and 5' regions). Although gene disruption is relatively quick because few DNA cloning steps are involved, it does have several drawbacks. One problem is that gene disruption results in a truncated peptide, which may still be functional, particularly if the internal fragment chosen is located toward the 3' region of the gene (Figure 4*A*). A second problem can arise from the fact that gene disruption creates a tandem repeat, which can be unstable under nonselective conditions. In our laboratory a strain in which the adenylate cyclase gene (*acyA*) was disrupted was only stable as long as hygromycin B was present in the medium. As soon as this selection pressure was removed, the disruption strains, which showed a significant growth defect, were outgrown by revertants, which had jettisoned the selection marker (B. Liebmann & A.A. Brakhage, unpublished observation). This result suggests that recombination is a relatively frequent event in *A. **fumigatus*** and argues against using gene disruption for studying gene function. In addition, gene disruption might not be feasible for small genes because relatively large internal fragments (0.5–1.0 kb) are required in order to achieve a reasonable gene disruption efficiency. This might simply not be possible for small genes.

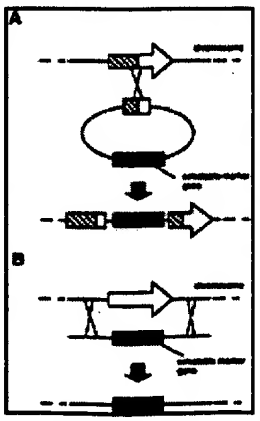

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Figure 4. (*A*) Schematic representation of a gene disruption event. The *open arrow* marks a gene of interest on a fungal chromosome. The *dark hatched box* represents a selectable marker gene on a plasmid. Following a single homologous recombination, the plasmid integrates in the gene of interest causing a disruption of the gene. (*B*) Schematic representation of a gene deletion event. The *open arrow* marks a region of interest on a fungal chromosome. The *hatched box* represents a selectable marker gene. Following a double homologous recombination, the region of interest is replaced by the selectable marker gene.

As a result, most publications report using gene deletion rather than gene disruption to study gene function in *A. **fumigatus*** (31). The method of choice for studying gene function is called gene deletion or gene knock-out (Figure 4*B*). Here, a plasmid is constructed in which the two regions flanking the gene of interest are separated by a selection marker. The flanking regions should be at least 1.0 kb in length in order to achieve a reasonably high recombination efficiency. The vector is linearized (and dephosphorylated) prior to transformation in order to prevent ectopic integrations and to promote double homologous recombination. Recombination events between the homologous flanking regions lead to the replacement of the chromosomal gene by the selection marker. This is a "clean" method and there is no risk of reversion because the gene is completely removed. A number of cloning steps are required to construct the deletion vector. Also, the bigger the flanking regions are the higher the recombination efficiency is, meaning that fewer transformants need to be screened in order to find one with the required gene deletion. Chaveroche et al. (22) reported a new method for generating gene deletions in *A. nidulans*. We have shown that this method also works for *A. **fumigatus*** (48A) (Figure 5). The method is based on the ability of *E. coli* strains expressing the λ *red* genes to carry out recombination with >50 bp of flanking sequence. A similar method making use of the *E. coli rec* genes was described by Zhang et al. (88) [reviewed in (59)]. These methods can be used to replace the gene of interest on a cosmid with a selectable marker. The resulting cosmids have large flanking regions and lead to high-efficiency homologous recombination. This means that fewer transformants need to be screened in order to detect a strain carrying a deletion in the relevant gene. At the moment, the only selectable marker available for this system is the *pyrG* gene of *A. **fumigatus***, which encodes orotidine-5'-monophosphate decarboxylase. The addition of other selectable markers will make this method even more powerful and will mean that *A. **fumigatus*** deletion strains can be generated more quickly and more easily than before.

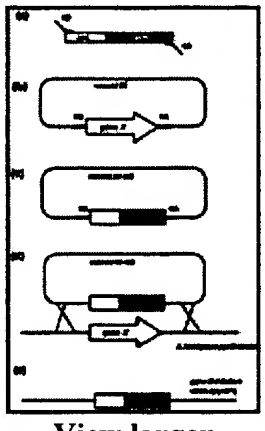

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Figure 5. Schematic representation of the ET-cloning technique (22, 48A). (*a*) A region encoding a bifunctional marker (*zeo* and *pyrG*) is amplified by PCR using primers that contain approximately 60 bp of sequence flanking the gene of interest (gene X). (*b*) An *Escherichia coli* strain carrying a cosmid (cosmid X1) that contains the gene X is transformed with the PCR product by electroporation. The expression of the λ *red* operon in the *E. coli* cells results in homologous recombination between the cosmid and the flanking sequences on the PCR product. (*c*) Cosmids in which the gene X has been replaced by the bifunctional marker can be selected for zeocin resistance in addition to the marker previously present on the cosmid. (*d*) The cosmid X1-KO is used to transform an *A. fumigatus pyrG*⁻ strain. The large flanking regions lead to a high rate of homologous recombination so that fewer transformants need to be screened. (*e*) The method produces deletion strains that are uracil prototrophic.

Sometimes gene deletions can result in a lethal phenotype. In such cases no transformants will be

obtained and it is difficult to study the effect of the gene. A solution to this problem used for e.g., *A. nidulans* can be to place the gene under the control of an inducible promoter such as the *A. nidulans alcA* gene promoter (Figure 6). For transformation a construct similar to the deletion construct is required, except that in addition to the selection marker the gene of interest is placed under the control of an inducible promoter that is located between the two flanking regions. In *A. nidulans* the *alcA* promoter can be induced by e.g., L-threonine and is repressed by glucose. In this manner transformants can be selected under conditions where the gene is active and the effect of a nonfunctional gene can be observed under other conditions. One drawback of this system is that the *alcA* promoter is slightly "leaky," allowing a small amount of expression even under repressing conditions. To date no strong inducible promoters are known for *A. **fumigatus***. This problem needs to be addressed.

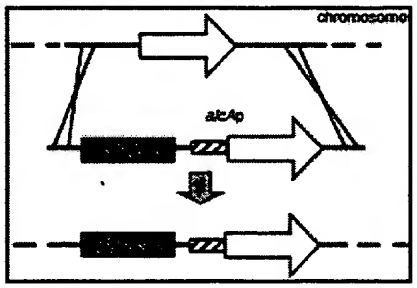

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Figure 6. Schematic representation of a method to artificially induce a gene of interest via the *alcA* promoter region. The *open arrow* marks a gene of interest on a fungal chromosome. The *dark hatched box* represents a selectable marker gene. Following a double homologous recombination, the gene of interest is replaced by a copy of the same gene under the control of the *alcA* promoter and the selectable marker gene. In this strain the gene of interest can be induced by carbon sources such as threonine and repressed by glucose. It is unlikely, however, that the *alcA* promoter without the presence of the *alcR* gene is active in *A. **fumigatus***.

Reporter Gene Technology

What mechanisms regulate gene expression in *A. **fumigatus***? More specifically, how are genes involved in virulence regulated? Answers to these questions would add significantly to our understanding of *A. **fumigatus*** and the factors responsible for making it an opportunistic pathogen. In other organisms reporter genes have been established as important genetic tools for analyzing the expression of particular genes under various conditions (12, 50, 66, 78, 83). Reporter genes have also been used to identify regions in the upstream sequence of a gene, which are important for regulating gene expression (e.g., 76, 86). Application of the ß-galactosidase-encoding *E. coli lacZ* gene in *A. **fumigatus*** was demonstrated for the first time by Smith et al. (74). They fused the *A. **fumigatus*** alkaline protease (*alp*) gene with the *E. coli lacZ* gene to demonstrate that the *alp* gene is expressed during lung colonization in a murine infection model. An example showing the use of the *lacZ* gene of *E. coli* in *A. **fumigatus*** is shown in Figure 7*A*. A reporter gene used extensively in *A. nidulans* (66) is the *uidA* gene encoding ß-glucuronidase. It could also be functional in *A. **fumigatus*** (31).

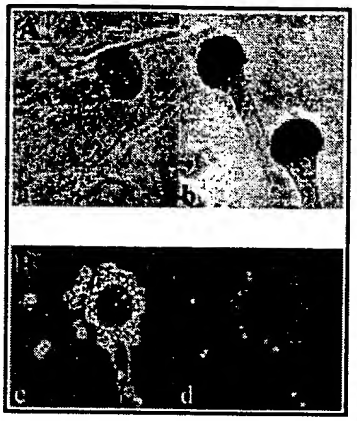

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Figure 7. Reporter gene systems in ***Aspergillus fumigatus***. (*A*) The *Escherichia coli lacZ* gene can be used as a reporter gene in *A. **fumigatus***. This can be visualized by addition of x-gal to fungal conidiophores. *Panel a*: The wild type of *A. **fumigatus*** shows no ß-galactosidase activity. *Panel b*: Fusion of the *lacZ* gene with a developmentally regulated gene results in a specific expression pattern (S. Gattung & A.A. Brakhage, unpublished observation). (*B*) The *egfp* gene encoding the enhanced green fluorescent protein has been used successfully to study gene expression patterns in *A. **fumigatus*** (50). *Panel c* shows the light microscopy image of a fungal conidiophore. *Panel d* shows the same conidiophore using a filter for green fluorescence. In this *A. **fumigatus*** strain the *egfp* gene is fused with a developmentally regulated gene, resulting in the observed expression pattern (K. Langfelder, unpublished result).

Recently Langfelder et al. (50) showed that another popular reporter gene, *egfp*, derived from the jellyfish *Aequorea victoria*, can also be used to study gene expression in *A. **fumigatus*** (Figure 7*B*). *egfp* encodes the enhanced green fluorescent protein (EGFP). *egfp* is especially well suited to studying protein localization and expression during infection because EGFP requires no additional cofactors. In the study the authors used *egfp* gene fusions to demonstrate differential expression and the expression of a virulence determinant during the infection process in a murine infection model. Taken together, these two reporter genes represent powerful tools for investigating gene expression in *A. **fumigatus*** and should provide new insights into the regulation of potential virulence determinants.

Ultimately the aim is to understand all the mechanisms controlling the expression of a gene. This includes a detailed analysis of the gene's upstream region, potential regulatory elements, and proteins binding to these elements. It is possible to use reporter genes, such as *lacZ*, to measure promoter activity (more difficult with *egfp* than with *lacZ*) by fusing the upstream region of the gene of interest with *lacZ*. In order to compare different upstream regions, possibly with deletions and/or point mutations, it is essential to integrate the constructs at a defined chromosomal locus. A system allowing the integration at the *pyrG* locus of *A. **fumigatus*** was developed by Weidner et al. (85) (Figure 8). The method relies upon using two mutant alleles of the *pyrG* gene, *pyrG1* and *pyrG2*, neither of which encodes a functional protein. The mutations in *pyrG1* and *pyrG2* are located at opposite ends of the gene. A uracil-auxotrophic *A. **fumigatus*** strain bearing the *pyrG1* allele is transformed with a plasmid bearing the *pyrG2* allele, and the selected transformants are uracil prototroph (i.e., do not require additional uracil). Such strains will have a functional *pyrG* gene, which can either result from a gene conversion of the *pyrG1* mutation or by integration of the plasmid via single crossover at the *pyrG* locus (Figure 8). The latter case occurs in about 45% of transformants and results in a targeted integration of the plasmid at the *pyrG* locus (85). Another locus that could be used for targeting gene fusions is the *sC* locus (26).

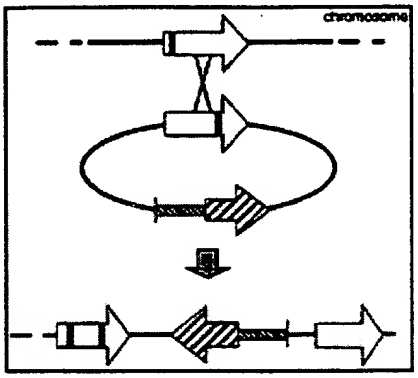

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Figure 8. Schematic representation of site-directed integration. The *open arrow* represents a selectable gene on one of the fungal chromosomes (e.g., *pyrG*). The same gene is present on the plasmid used for transformation. Both the chromosomal copy and the plasmid copy of the selectable gene carry different mutations (indicated by *black bars*). Neither copy encodes a functional protein. Integration of the plasmid at the site of the selectable gene (e.g., *pyrG*) by single homologous recombination results in two copies of the gene, one of which is intact while the other carries both mutations. The *hatched arrow* marks a gene of interest, e.g., a reporter gene fusion with a promoter region to be analyzed.

A useful tool that can be used in conjunction with the techniques described above is a method for generating point mutations in promoter regions by PCR. The method pioneered by Higuchi et al. (44) uses two sets of oligonucleotide primers that bind at each end of the promoter region to be studied as well as in the region where one or several point mutations are to be made. The oligonucleotide primers binding at the mutation site overlap and are oriented in opposite directions. They must be designed to contain the necessary mutation. Using these primers two independent PCR reactions are carried out. Both products will carry the point mutation. The PCR products are purified and mixed together. The mixture of PCR products is then denatured by heating to 94°C and allowed to cool slowly to room temperature. As the two PCR products overlap slightly a small proportion of fragments will form heterologous pairs (by far the greater number will bind to their complementary sequence). A second PCR reaction using the primers lying on the outside of the template will amplify only those PCR fragments that have formed heterologous pairs. The result of this PCR will be a full-length product that contains the point mutation required. This can then be cloned upstream of the *lacZ* reporter gene and transformed into *A. **fumigatus*** to measure the effect on gene expression. Although this method has not yet been described for *A. **fumigatus***, it was used in *A. nidulans*, e.g., to analyze the promoter of the *hapB* gene (76).

Applied Transformation Technologies

SIGNATURE TAGGED MUTAGENESIS

Signature tagged mutagenesis (STM) is a method used to screen for genes essential for infectious growth in pathogenic bacteria (42, 43). In STM large numbers of mutants are created by insertional mutagenesis using, e.g., REMI. Each DNA molecule inserted carries an individual signature tag, which can be identified by hybridization. Brown et al. (16) adapted the method to study virulence determinants in *A. **fumigatus***. They created several pools of mutants and used these to infect immunosuppressed mice. The mutant strains were then reisolated from the lungs and compared with the strains used to infect the mice. Strains that could not be reisolated potentially carried a deletion in a gene essential for infectious growth of *A. **fumigatus***. Using this technique Brown et al. (16) were able to identify the *A. **fumigatus** pabaA* gene that encodes *para*-aminobenzoic acid synthetase. Furthermore, they showed that the gene is essential for invasive growth. However, *pabaA* can hardly be termed a virulence factor, and no virulence factors have

been identified to date using STM. One explanation could be that *A. **fumigatus*** does not have any major virulence factors but does have a number of physiological characteristics (or "virulence determinants") such as the *pksP* gene enabling it to be an opportunistic pathogen (52). Alternatively, the STM method described here is simply not sensitive enough to identify genes that might have only a small effect on virulence. It is possible that a coinfection model of aspergillosis would lead to better results (51), allowing this method to be used more effectively on *A. **fumigatus***. For coinfection mice are infected simultaneously with equal doses of wild-type and mutant strains, resulting in the death of the mice. Fungal cells are then isolated from the mice and the ratio of wild-type to mutant colony-forming units is observed. A significant difference would indicate that the mutant strain is defective in some function involved in pathogenicity.

IN VIVO EXPRESSION TECHNOLOGY

In vivo expression technology (IVET) was originally developed in bacteria to identify promoter regions that would lead to efficient expression during the infection process (54). This technique could be adapted for use in *A. **fumigatus***, as was accomplished by Staib et al. (75) for *Candida albicans*. Two different approaches can be used for IVET. The first approach investigates a specific promoter region, as was done with *C. albicans* (75). In this case the promoter of interest is fused in frame with a marker gene that allows the identification of strains expressing this reporter gene during infection. Possible markers for *A. **fumigatus*** are the genes *pyrG*, *pabaA*, and *sC* in the relevant deletion strains. Following transformation of the relevant strain (using a different selectable marker), transformants are used in an *A. **fumigatus*** infection model. Strains grow only if the promoter fused with the marker gene is active during the infection process. Promoters that are not active under normal conditions but are specifically activated during infection could be involved in virulence.

The second approach is to generate a library of *A. **fumigatus*** mutants in which random fragments of fungal DNA are fused with the relevant marker gene (31). The strains are used in an infection model of aspergillosis. Again, only those strains can survive where the DNA region fused with the marker gene leads to expression during the infection process. If such DNA fragments do not lead to expression under normal conditions, they could indicate that the attached gene is involved in the infection process. It is advisable to use targeted integration (e.g., to the chromosomal *pyrG* locus) for both approaches to ensure that no external factors influence marker expression. A possible problem could result from the sensitivity of this method because it distinguishes only between growth and no growth.

GENOMICS

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The complete genome of *A. **fumigatus*** is currently being sequenced (see http://www.tigr.org/; http://www.sanger.ac.uk/). Based on preliminary sequencing data, the genome of *A. **fumigatus*** was estimated to consist of 28.7 Mb (http://www.tigr.org/). In parallel to the progress of the sequencing, data have been already released. The available sequence data can be expected to have a major impact on *A. **fumigatus*** research. These data will allow comparative genomics to answer questions about the differences

between pathogenic and nonpathogenic ***Aspergillus*** species. Furthermore, they will allow large-scale biological studies (transcriptome and proteome analyses) to identify genes/proteins that are specifically transcribed/produced during the infectious process and may represent factors unique to *A. **fumigatus***. The genome information can also be expected to help to identify targets for the generation of novel drugs.

CONCLUSION

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As we have shown here, the past years have seen a dramatic increase in the range of tools available for studying the opportunistic fungal pathogen *A. **fumigatus***. Methods have been established for transforming the fungus. These can be used in conjunction with the existing selection systems for *A. **fumigatus*** or with systems that are currently used in other fungi such as *A. nidulans* and *A. niger* and can be adapted for *A. **fumigatus***. It might be possible to establish a library of auxotrophic *A. **fumigatus*** mutants in order to facilitate working with the parasexuality of *A. **fumigatus***. Recently developed tools for *A. **fumigatus*** include techniques for the rapid generation of deletion strains and new reporter genes. Working with these tools, improving the techniques available, and developing new approaches will enable researchers to carry out a detailed study of *A. **fumigatus***. The genomic sequence of *A. **fumigatus*** that will soon become available will provide the *A. **fumigatus*** community with an additional powerful tool and allow for a better understanding of the fungus in general and factors involved in virulence in particular. Eventually this will allow the identification of new targets for antifungal substances and the development of new treatments to prevent the high mortality rates associated with a systemic aspergillosis.

ACKNOWLEDGMENTS

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BIOLOGICAL WEAPONS—A PRIMER FOR MICROBIOLOGISTS[1]

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KEY WORDS: personal protection, incident response, decontamination

ABSTRACT

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Biological weapons are not new. Biological agents have been used as instruments of warfare and terror for thousands of years to produce fear and harm in humans, animals, and plants. Because they are invisible, silent, odorless, and tasteless, biological agents may be used as an ultimate weapon—easy to disperse and inexpensive to produce. Individuals in a laboratory or research environment can be protected against potentially hazardous biological agents by using engineering controls, good laboratory and microbiological techniques, personal protective equipment, decontamination procedures, and common sense. In the field or during a response to an incident, only personal protective measures, equipment, and decontamination procedures may be available. In either scenario, an immediate evaluation of the situation is foremost, applying risk management procedures to control the risks affecting health, safety, and the environment. The microbiologist and biological safety professional can provide a practical assessment of the biological weapons incident to responsible officials in order to help address microbiological and safety issues, minimize fear and concerns of those responding to the

incident, and help manage individuals potentially exposed to a threat agent.

INTRODUCTION

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Biological weapons derived from biological material(s) are considered weapons of mass destruction or, more appropriately, as weapons of mass casualty. A biological weapon can be considered a four-component system composed of a payload, munition, delivery system, and dispersion system (20). The payload is biological material consisting of an infectious agent (a pathogen) or a toxin produced by bacteria, plants, or animals. The munition serves to containerize the payload to maintain its potency during delivery. The delivery system can be a missile, vehicle (aircraft, boat, automobile, or truck), or an artillery shell to transport the payload to a susceptible target. The dispersion system, provided by an explosive force or spray mechanism, ensures dissemination of the payload at the intended target.

Although the list of potential agents is numerous, those agents that could cause mass casualties by the aerosol route of exposure are considerably smaller (8, 10, 11, 12, 15, 21, 27, 41, 57, 80). Infectious biological payloads that could potentially be used include those causing anthrax (*Bacillus anthracis*), plague (*Yersinia pestis*), tularemia (*Francisella tularensis*), equine encephalitides (Venezuelan equine encephalitis, eastern equine encephalitis, and western equine encephalitis viruses), hemorrhagic fevers (arenaviruses, filoviruses, flaviviruses, and bunyaviruses), and smallpox (variola virus). Toxins include botulinum toxin from *Clostridium botulinum*; ricin toxin from the castor bean *Ricinus communis*; trichothecene mycotoxins from ***Fusarium***, *Myrotecium*, *Trichoderma*, *Stachybotrys*, and other filamentous fungi; staphylococcal enterotoxins from *Staphylococcus aureus*; and toxins from marine organisms such as dinoflagellates, shellfish, and blue-green algae. If these agents are delivered successfully to a susceptible host, a lethal or incapacitating outcome will occur, depending upon the agent. For instance, the agents causing anthrax and plague will most likely result in death of the target host. *Coxiella burnetii* (the agent of Q-fever), staphylococcal enterotoxin B, and Venezuelan equine encephalitis virus are considered incapacitating agents. In a military context, incapacitating agents may be in a certain sense more effective because the unit will not be able to perform their mission and casualties will consume scarce medical and evacuation assets.

Both lethal and incapacitating agents could have an adverse impact on the civilian healthcare delivery system in a biological terrorism scenario. Potential manifestations include terror in the affected population and in medical care personnel; an overwhelming number of casualties placing demands for ICU care or special medications; a need for personal protection in medical care settings, clinical laboratories, and autopsy suites; and problems with handling remains. Employing standard precautions or barrier nursing techniques (23), depending on the agent, can provide appropriate (or adequate) protection to healthcare providers against biological agents. However, additional precautions such as aerosol, droplet, or contact protection are recommended in instances where smallpox, *Y. pestis* (plague), the hemorrhagic fever viruses, or T-2 mycotoxin are suspected. There may be a hazard of person-to-person transmission, transmission by direct contact

with blood or body fluids, or dermal activity (13, 27, 28, 31, 32, 65, 86).

HISTORICAL ASPECTS

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Biological weapons are not new, but the technologies of production and delivery have been developed and perfected by nations during the twentieth century. For thousands of years, biological agents have been available as instruments of warfare and terror (bioterrorism) to produce fear and harm in a vulnerable population. In addition to the general human population as possible targets, animals and plants have also been possible targets for those desiring to use these agents. To initiate an epidemic of plague during the fourteenth century siege of Kaffa, the attacking Tatar force catapulted the bodies of their deceased soldiers into the city. During the 1754–1767 French and Indian War, the British contemplated the deliberate use of smallpox against Native American tribes. An opportunity to exercise the plan using fomites occurred during an outbreak of smallpox at Fort Pitt. On June 24, 1763, blankets and a handkerchief were given to the immunologically naïve Native Americans in an attempt to "reduce" tribes hostile to the British (10).

Germany developed plans during World War I to contaminate animal feed and infect livestock for export to Allied forces. They also planned to infect sheep from Romania with *B. anthracis* (anthrax) and *Burkholderia mallei* (glanders) for export to Russia. German saboteurs operating in Mesopotamia allegedly were to use *B. mallei* to inoculate 4500 mules and infect horses of the French cavalry in France (17). Livestock in Argentina, destined for export to Allied forces during 1917–1918, were infected with *B. anthracis* and *B. mallei*, resulting in the death of more than 200 mules (10).

Japan conducted biological warfare experiments in Manchuria from 1932 to 1945 (88). At the infamous Unit 731, a biological warfare research facility near the town of Ping Fan, prisoners were infected with *B. anthracis*, *Neisseria meningitidis*, *Shigella* spp., *B. mallei*, *Salmonella typhosa*, *Vibrio cholerae*, *Y. pestis*, smallpox virus, and other disease-causing agents (34). In addition, a number of Chinese cities were attacked with biological warfare agents. The Japanese contaminated water supplies and food items with *B. anthracis*, *Shigella* spp., *Salmonella* spp., *V. cholerae*, and *Y. pestis*. Cultures were also tossed into homes and sprayed from aircraft. Potentially infected fleas were harvested in the laboratory and as many as 15 million fleas were released from aircraft per attack. Because of inadequate preparation, training, and/or lack of proper equipment by the Japanese, the Chekiang Campaign in 1942 reportedly led to about 10,000 biological casualties and 1,700 deaths among the Japanese troops, most from cholera and some from dysentery and plague (89).

Prisoners in Nazi concentration camps were forcibly infected with *Rickettsia prowazekii*, *Rickettsia mooseri*, hepatitis A virus, and *Plasmodium* spp., and also treated with investigational vaccines and drugs (48). The Germans polluted a large reservoir in northwestern Bohemia with sewage in May 1945 (76), the only known tactical use of biological warfare by the Germans. For potential retaliatory use in response to a German biological attack, the British developed

biological warfare capabilities by conducting bomb experiments with weaponized spores of *B. anthracis* on Gruinard Island near the coast of Scotland during 1941 and 1942. Viable anthrax spores persisted for 45 years after World War II until the island was decontaminated with formaldehyde and seawater in 1986 (44).

The United States' offensive biological warfare program began in April 1943 at Camp Detrick, Maryland (renamed Fort Detrick in 1956), with testing sites at Horn Island, Mississippi, and Granite Peak, Utah (22). Experiments were conducted with *B. anthracis* and *Brucella suis*. A new production facility was constructed at Pine Bluff, Arkansas (45), during the Korean War (1950–1953) to meet projected demands. Human experiments were conducted at Camp Detrick with nonlethal agents in 1955. Exposure of volunteers to biological munitions containing *F. tularensis* and *C. burnetii* occurred in a 1-million-liter spherical aerosolization chamber. Volunteers put their face up to an opening in a portal for dosage studies. Studies were designed and conducted to determine the volunteers' vulnerability to aerosolized pathogens and the efficacy of vaccines, prophylaxis, and therapies under development. Additional studies were done with the simulants *Aspergillus fumigatus*, *B. subtilis* var. *globigii*, and *Serratia marcescens* to determine production and storage techniques, aerosolization methods, the behavior of aerosols over large geographic areas, and the effects of solar irradiation and climatic conditions on the viability of aerosolized organisms.

During 1952–1953, Moscow, Peking, and Pyongyang alleged that US Armed Forces used biological weapons against targets in North Korea and China. Although these allegations were not conclusively proven true or false by the available evidence, and were denied by the US Government, they caused the United States a loss of international good will. Belief in the US use of biological weapons during this period of time still persists. The events in Brazil during 1957–1963 are reminiscent of the technique used by the British against Native American Indians in the eighteenth century. In the 1969 trial, the Brazilian Ministry of the Interior disclosed evidence on the deliberate use of the agents smallpox, chickenpox, tuberculosis, influenza, and measles on several Indian tribes in the Mato Grosso. These agents were allegedly introduced in order to clear the Indian tribes from valuable rubber land. The *South China Morning Post* reported that Chinese officials accused the United States of plotting the cholera epidemic in the southeast province of Kwantung Province in the summer of 1961. The US Department of State denied this accusation (77).

During the 1962–1968 war in Indo-China, Viet Cong rebels used a crude form of biological weapon. They used spear traps and hidden bamboo spikes ("pungi sticks") tipped with animal or human excrement as a booby trap hazard for US forces in Vietnam. These weapons produced casualties by causing percutaneous infection in victims after they came in contact with the spear traps and spikes (18, 78). US allegations of the use of aerosolized trichothecene mycotoxins ("yellow rain") in Laos (1975–1981), Kampuchea (1979–1981), and Afghanistan (1979–1981) by Soviet armed forces and their surrogates is unsubstantiated for many reasons (10). An interesting point must be raised regarding the allegations of biological weapons use by various factions or governments. Conclusive evidence is less likely to be found the longer the time period between a report of the alleged use of a biological weapon and an investigation. Whatever the belief, there is

some uncertainty of their use (77).

An attempted assassination in 1978 of the Bulgarian exile Vladimir Kostov in Paris, France used ricin (the toxin extracted from castor beans). A ricin-containing pellet was discharged from an umbrella gun into his back, but because of the heavy clothing he was wearing, the pellet did not penetrate deep enough in his body for the wax pellet coat to melt. The Bulgarian exile Georgi Markov was assassinated 10 days later in London with a ricin-filled polycarbonate ball (46). An umbrella gun discharged the pellet into the subcutaneous tissue of his leg while he was waiting at a metro stop. Despite care administered during his hospitalization, he died three days later (19).

Use, or the potential use, of biological weapons during the past 20 years has been exhaustively publicized, especially since the discovery of an extensive biological warfare program in Iraq after the Gulf War of 1991. Coupled with knowledge of the covert program in the former Soviet Union (2, 3), considerable public attention in the United States has been focused on the use and consequences of a biological weapons encounter. The Rajneeshee Cult, an Indian religious group, contaminated restaurant salad bars in Oregon in 1984 with *Salmonella typhimurium*, and about 751 citizens were infected. The cult's motivation was to incapacitate voters in order to win a local election and to seize political control of Dalles and Wasco counties (74, 85). In 1991, the Minnesota Patriots Council, a group consisting of anti-government tax protesters, planned to inoculate Internal Revenue Service officials, a US Deputy Marshal, and local law enforcement officials through the skin with ricin. Their objective was to cause harm to the federal government and obtain personal revenge (85). Larry Wayne Harris wanted to alert Americans to the Iraqi biological warfare threat and sought a separate homeland for whites in the United States. He had links to Christian Identity and the Aryan Nation, a white supremacist group. Harris made vague threats against US federal officials on behalf of right-wing "patriot" groups. He obtained the *B. anthracis* vaccine strain, *Y. pestis*, and reportedly several other bacteria and discussed the dissemination of biological warfare agents by means of crop duster aircraft and other methods. Harris was arrested in 1998 after he made threatening remarks to US officials and openly talked about biological warfare terrorism (85).

Beyond the shores of the United States, the Aum Shinrikyo (Aum Supreme Truth) Cult sought to establish a theocratic state in Japan with a charismatic, power hungry leader named Shoko Asahara (75). Their objective was to prove an apocalyptic prophecy, eliminate enemies and rivals, halt an adverse court ruling regarding a real estate dispute in Matsumoto in 1994, and seize control of the Japanese government. In 1995, they disseminated the chemical agent sarin (inhibits acetylcholinesterase, thereby disrupting nerve impulse transmission) in the Tokyo subway system. Their multiple attacks with many chemical agents such as hydrogen cyanide (prevents the normal utilization of oxygen) and VX (mechanism of action similar to that of sarin), and assassinations in other areas of Japan resulted in the injury of more than 1000 persons and the deaths of at least 20 (85). These chemical agents can be incapacitating or lethal, depending upon the dosage. Targets of the Aum Shinrikyo Cult were mass civilian populations, individuals opposed to their ideology, and judges ruling against and police investigating the cult. They attempted to obtain Ebola virus from Zaire, Africa, in 1993, and in 1994 discussed the possibility of using Ebola virus as a biological weapon. The cult also cultured and experimented with the agents of anthrax, Q-fever,

cholera, and botulinum toxin (54, 85).

PROPERTIES OF BIOLOGICAL AGENTS

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Biological agents may be used, perhaps as an ultimate weapon, because of several characteristics valued by the perpetrator. Aerosols of biological agents are invisible, silent, odorless, tasteless, and are relatively easily dispersed without detection. They are relatively inexpensive to produce. It is estimated that the cost would be about 0.05% the cost of a conventional weapon to produce a similar amount of mass casualties per square kilometer. In addition, their production follows common fermentation technology that is used for the production of some antibiotics, vaccines, foods, and beverages. Basic, commonly available technology is available for their delivery, such as spray devices from an airplane, boat, or car, over large areas. Terrain, equipment, and infrastructure are usually spared because explosives are not normally used for the delivery of biological agents. Because of this simplicity, it is not difficult for users to tailor their arsenal to fit their needs. Biological weapons can be used in combination with other weapons to create fear, terror, and panic in a population, producing large numbers of casualties. The consequences of their use are many. They may rapidly overwhelm medical resources, and the perpetrators could escape before any effects are even noticed because most agents have incubation times of several hours to days. In addition, the use of an endemic agent may cause confusion because of the inability to differentiate a biological warfare attack from a natural epidemic, and, although limited, a potential exists for secondary or tertiary transmission.

Where can an individual or group obtain biological agents for use in biological warfare or develop biological weapons, and how can these agents be delivered to a susceptible target? Biological agents can be obtained from multiple culture collections, universities, commercial chemical and biological supply houses, foreign laboratories, and from field samples or clinical specimens. Aerosol delivery is considered optimal if the particle size is between 1 and 5 μm because particles with these characteristics will settle in the lower respiratory tract and be undetectable by our senses. Smaller particles will be exhaled because of the aerodynamics of particle flow through the respiratory tract. Larger particles will settle on environmental surfaces or on the upper respiratory tract, allowing mucocilliary clearance. Delivery of a biological agent by an explosive device is less effective because the heat and light from an explosion may inactivate the agent. Production of particles of 1–5 μm in size is inefficient in an explosion. Delivery methods can include an open-air line source delivery system where a spray device is attached to a moving conveyance. An open-air point source delivery system employs a stationary device such as a sprayer, bombs, or bomblets (a device with a physical guidance system designed to disseminate the biological agent upon impact or at a predetermined altitude above the ground). Limited air-delivery applications include spray devices, bombs, or bomblets. Delivery in water supplies may not be effective because of the dilution factor and because water purification methods such as chlorine treatment, coagulation and flocculation methods, and reverse osmosis systems tend to inactivate microbial agents. There must be increased vigilance, however, in some circumstances because the effectiveness of delivery of a biological agent(s) in water can be increased by its delivery at or

near the source of consumption. Biological agents can also be delivered by direct application as in the case of assassination by pellets (46) or flechettes (a dart-shaped projectile) (15).

Personal Protection

How can we protect ourselves against biological warfare agents? The primary means of protecting both persons working with potentially hazardous biological materials and the environment in a laboratory or research environment may be use of engineering controls, combined with proper good laboratory and microbiological techniques, and common sense. Equally important is the use of certain personal protective equipment and measures (such as an occupational health program) and decontamination procedures. Implementing protective measures and associated procedures depends upon both the situation and their availability. Standard precautions (designed to reduce the risk of transmission of microorganisms from both recognized and unrecognized sources of infection) and protection against transmission by contact, droplet, and airborne vectors, where appropriate, should be employed by all laboratory workers to prevent their exposure to potentially hazardous materials (37). Standard precautions (29, 30) combine the major features of universal precautions (blood and body fluid precautions designed to reduce the risk of transmission of bloodborne pathogens) and body substance isolation (designed to reduce the risk of transmission of pathogens from moist body substances).

Engineering controls (82) refer to those barriers applied at the point of origin of the hazard. The most important primary barriers are biological safety cabinets, some form of animal cage containment (cage tops with filters and laminar flow enclosures), and positive-pressure protective suits. Biological safety cabinets (e.g., Class I, II, and III cabinets) are the most effective, as well as the most commonly used, primary containment devices in laboratories working with infectious agents (61, 62). Incorporating charcoal filters in the exhaust system of cabinets makes them suitable for use with toxins suspended in volatile material. In situations where the agent is highly hazardous (and where there are no other protective measures, such as a vaccine against the agent), laboratory workers are physically protected by a positive-pressure protective suit, a type of primary barrier, in biosafety level-4 laboratories.

Secondary barriers of protection against biological agents include design features that protect individuals inside and outside the facility. These barriers vary depending upon the hazards presented by the agents and materials and the laboratory procedures used in the facility (63). Secondary barriers may include physical barriers in the laboratory or research facility (such as limited access, separate room entry areas, personnel airlocks, or change rooms), directional air flow of nonrecirculated air, and discharge of exhaust air remote from occupied areas and air intakes. Exhaust air must be filtered through one or more high-efficiency particulate air (HEPA) filters in series with certain infectious agents or at biosafety level 4. HEPA filters are constructed of paper-thin sheets of borosilicate medium, pleated to increase surface area with aluminum separators for added stability, and affixed to a frame. A HEPA filter removes particulate material the size of 0.3 μm or greater with a minimum efficiency of 99.97% (59, 83). Other forces, such as electrostatic charge and the effects of filtration velocity, impaction, and entrapment, affect filtration efficiency of particles smaller than 0.3 μm. Additional barriers include nonionizing ultraviolet (uv) light irradiation (which is lethal to a wide variety of bacteria and viruses, but its

effectiveness depends on the intensity and length of exposure); sinks for handwashing; screened or sealed windows; equipment for decontamination and disposal of hazardous materials; work surfaces amenable to cleaning, housekeeping, and decontamination; and personal protective equipment (PPE).

PPE includes clothing and equipment used to protect individuals in a laboratory or research environment from contact with infectious or toxic materials or physical hazards. The appropriate PPE for an activity depends upon the operations conducted and the potential hazards associated with the activity. It must be emphasized that PPE, although an important item of personal protection, only serves as a secondary barrier against hazards in the laboratory or research environment. Proper PPE must be carefully chosen to mitigate the hazards presented by the agents and procedures used. To assist in the selection of appropriate PPE, workers should consult agent summary statements (64), agent manuals (9), material safety data sheets (when handling hazardous or potentially hazardous chemicals), facility standard operating procedures, and persons knowledgeable about the associated hazards, such as facility safety personnel. As a minimum, with consideration of the risks involved, PPE may include street attire protected by a full-length, long-sleeved, fully fastened laboratory coat, gown, or smock; closed-toe shoes; eye protection; ear protection; molded "surgical type" masks (filtering facepiece); appropriate gloves ("examination" or "surgical" type depending upon the need for sterile procedures); and HEPA-filtered respirators. Although HEPA filters used in respirators are not certified by the National Institute of Occupational Safety and Health (NIOSH) for use in a biological environment (65), these filters have been successfully used to protect personnel for many years.

Personal protective measures also include elements of an occupational health program. One element is medical surveillance, during which a physician determines if an individual is medically qualified to work with potential occupational hazards. Another element is the collection and storage of baseline serum samples, when appropriate, from individuals handling certain hazardous agents, or those participating in a special immunizations program. The occupational health program should evaluate the physical and mental suitability of personnel for duty assignment in areas where certain hazardous agents are handled. Another element of the program is to vaccinate at-risk personnel, those who may be occupationally exposed to certain agents. If a safe vaccine is available for the agent(s) being used and is known to protect against the agent(s), it should be provided for those individuals working directly with the agent(s) and for other at-risk personnel (such as facility maintenance, animal care, and safety personnel). The program should also include a mechanism for the immediate reporting of potential exposures to agents, such as mishaps, spills, or other accidents. It is extremely important to also report any illness that may be associated with working with these agents, no matter how remote the possibility of exposure may seem or even if an incident cannot be associated with the illness. Each exposure, or potential exposure, should be evaluated by appropriate medical staff and, if required, treated. Medical treatment of individuals potentially exposed to biosafety level 2, 3, or 4 agents can be safely accomplished by standard or transmission-based (airborne, droplet, or contact) precautions where appropriate (31, 32). The availability of a quarantine facility with a capability for isolation and medical care of personnel with a potential or known laboratory-associated exposure is required for Army facilities handling biosafety level 4 (maximum containment) agents (84).

DECONTAMINATION

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Decontamination procedures in a laboratory or research environment will vary depending upon the capabilities of the facility and the agent(s) being used (7, 20, 47, 68, 69). Decontamination is disinfection or sterilization of articles containing etiologic agents (microorganisms or toxins) to make them safe for use or disposal. Disinfection is the selective elimination of certain undesirable microorganisms to prevent their transmission. Sterilization is the complete destruction of microbial life (5, 20, 69).

Decontamination procedures can be discussed from two approaches: surface decontamination and area (space) decontamination. For surface decontamination, the effectiveness of a decontaminant depends upon decontaminant concentration, the concentration of the agent, type of agent, time of contact, and the environmental conditions (38, 43, 70, 87). There are a number of general groups of decontaminants such as alcohols, halogens, quaternary ammonium compounds, phenolics, and glutaraldehyde. Many of these decontaminants (1) are only active against certain groups of microorganisms while inactive against others. These decontaminants are used primarily for the interior of safety cabinets, room surface washdowns, wiping off the exterior of certain items being removed from laboratories, and in chemical disinfectant showers used to disinfect a positive-pressure protective suit. Some of the halogen-containing decontaminants are corrosive. There is an association between autoclave corrosion and the use of halogenated disinfectants, particularly chlorine disinfectants. Chlorine may combine with organic materials during autoclaving. The resulting compound, or compounds, is corrosive in the autoclaves, drains, vents, and the central vacuum and receiver system that supports the autoclaves. The activity of chlorine-containing disinfectants, such as sodium hypochlorite solution, can be neutralized with sodium thiosulfate.

The second approach to decontamination is area or space decontamination, that is, decontamination of equipment or materials within enclosed spaces. This can be achieved by using a variety of gases (55). Ethylene oxide (epoxyethane, ETO) is a flammable and explosive gas, classified as both a mutagen and a carcinogen. The microbicidal activity of ETO is due to alkylation of sulfhydryl, amino, carboxy, phenolic, and hydroxyl groups in the spore or vegetative cell. The reaction of ETO with nucleic acids is the primary mechanism of its bactericidal and sporicidal activity. ETO is used because of its ability to inactivate most bacteria, molds, yeasts, and viruses, but its use is limited because of the many dangers mentioned. Propylene oxide (epoxy propane) hydrolyzes in the presence of moisture to form propylene glycol, which is nontoxic. Propylene glycol vapor (58) is odorless, tasteless, and nonirritating to the respiratory mucosa. The microbicidal mode of action of propylene oxide is the alkylation of DNA guanines resulting in single-strand breaks. Beta-propiolactone (BPL) is approximately 4000 times more active than ETO and 25 times more effective than formaldehyde. The microbiological activity of BPL is due to alkylation of DNA. However, there is limited use of this decontaminant because BPL lacks the ability to penetrate material and is carcinogenic in mice. Formaldehyde is more widely recognized as a fumigant for buildings and rooms (73, 79). Formaldehyde gas is capable of killing microorganisms and detoxifying *Clostridium botulinum* toxin. The microbicidal activity of

formaldehyde is due to denaturation of proteins. Ammonium bicarbonate can be used to neutralize formaldehyde gas. Although formaldehyde vapor is explosive at concentrations between 7.0%–73.0% by volume in air, these concentrations should not be reached if standard decontamination procedures (using 0.3–0.6 g/ft^3 of paraformaldehyde in the presence of 60%–90% relative humidity) are used. The most commonly recommended decontaminant for an area is formaldehyde. It is used to decontaminate biological safety cabinets (25), laboratory rooms, laboratory areas, and equipment in airlocks. Although widely used and recommended as a surface and area sterilant, formaldehyde is a safety hazard because it is a potential occupational carcinogen. In addition, it is a powerful reducing agent, has limited penetrating ability, and is potentially explosive. Environmental release of formaldehyde is also highly regulated. For these reasons, technologies that may provide alternative sterilants are emerging. One alternative is the powerful oxidant chlorine dioxide, which is an effective sterilant even at concentrations as low as 20 mg/l. A relative humidity of 50% or higher is optimal for sterilization. Another alternative, ozone, is not a new sterilant; it was used to sterilize the water supply of Lille, France, in 1899. It has potential as a sterilant for medical devices because it is highly oxidizing. The alternative gas plasma sterilization process uses radio frequency energy and hydrogen peroxide vapor to create a low-temperature hydrogen peroxide gas plasma to achieve relatively rapid sterilization. Radio waves break apart the hydrogen peroxide vapor into reactive species (free radicals), which form a gas plasma that interacts with and kills microorganisms. The advantage of this process is that the process temperature does not exceed 40°C. A relatively new alternative sterilization system is based on the vapor phase of hydrogen peroxide (6). The system provides a rapid, low-temperature technique that, because of its low toxicity, eliminates much of the potential public health hazard associated with decontaminants such as formaldehyde and ethylene oxide. In the cold sterilization process, 30% liquid hydrogen peroxide (300,000 ppm) is vaporized to yield 700–1200 ppm. The hydroxyl radical, a strong oxidant, is believed to have microbicidal activity through attack on membrane lipids, DNA, and other essential cell components. The hydrogen peroxide vapor is unstable and degrades to the nontoxic residues of water vapor and oxygen. Any sealable enclosure, such as small rooms, airlocks, biological safety cabinets, glove boxes, and isolation equipment, (up to 1,200 ft^3) can be sterilized. The process is effective at temperatures ranging from 4°C to 80°C. The vapor phase hydrogen peroxide sterilization system appears to be safe and is effective against a variety of microorganisms. The range of etiologic agents inactivated by various decontamination techniques is extensively documented (1, 7, 47, 55, 58, 68, 69, 73, 87).

PROTECTION DURING A RESPONSE TO AN INCIDENT

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During a response to an incident involving biological agents, or during work in the field, it is anticipated that only personal protective measures and equipment and decontamination procedures may be available to personnel. Protection against biological agents can usually be provided by PPE used to protect against hazardous chemicals. As in a laboratory or research environment, it is important to consider the hazard when selecting PPE in a field environment. The availability and procedures for the use of protective equipment by military personnel against biological agents in a combat environment is well documented (13, 24). Personnel trained in microbiology and biological safety are essential

resources in providing support to law enforcement agencies in response to an act(s) of bioterrorism or incident involving biological weapons. These professionals will be able to apply the principles of biosafety in a practical approach to the terrorism incident. An immediate evaluation of the situation is foremost and is accomplished by employing risk management procedures following the guidelines for conducting a risk assessment (4, 26, 33, 71, 81). Risk management is the systematic application of policies, practices, and resources to the assessment and control of risk affecting human health, human safety, and the environment. Risk assessment is an expression of potential loss in terms of hazard severity, accident probability, and exposure to the hazard. One must consider the severity of the hazard by assessing the expected consequence, which is defined as the degree of injury or occupational illness that could occur from the hazard. The probability of an accident or illness occurring after a given exposure to the hazard must also be determined. Finally, exposure to the hazard considers the number of persons exposed and the duration or frequency of the exposure. A risk assessment will provide guidance for choosing the appropriate PPE for responding personnel and subsequent management of those individuals potentially exposed to a threat agent. A risk assessment is never completed. It is a constant review of implemented procedures, policies, and plans. Risk assessment is a method for reducing all hazards to the minimal acceptable level.

Levels of protection for a hazardous chemical incident (Table 1) are categorized from levels A (maximum protection) through D (limited protection). Level A provides the highest level of respiratory, skin, and eye protection. Level B provides the highest level of respiratory protection but less skin protection. Level C is used when the concentration and identification of the air contaminant is known so that an appropriate NIOSH approved air-purifying respirator can be used. Level D protection can be used where the atmosphere is free of all known hazards and the tasks do not pose a splash, immersion, or potential respiratory hazard. Level D is comparable to PPE worn in a hospital laboratory, that is, a hospital gown or laboratory coat, goggles, surgical mask, and latex examination or surgical gloves. Depending on the hazard, protection afforded by Level D or Level C may be adequate for emergency response personnel (67) or in a field environment. Where the hazard lingers and is concentrated and may be volatile, protection against biological agents can be provided by employing standard precautions or barrier nursing techniques depending on the biological agent (23, 37, 65). Individuals will be protected against the potential of person-to-person transmission of the agent(s) and any environmental biological hazards. The rationale is that biological agents, in most situations, would most likely be dispersed from the area before the arrival of personnel (35, 66). However, additional precautions such as respiratory and cutaneous protection are recommended in certain instances where smallpox, *Y. pestis*, the hemorrhagic fever viruses, or T-2 mycotoxin are suspected, and where there may be a hazard of person-to-person transmission, transmission by direct contact with blood or body fluids, or dermal activity (27, 28, 49, 65, 86).

View this table: [in this window] [in a new window]	**Table 1. Levels of protection—hazardous chemical incident**

Protection against biological warfare agents during a response to an incident, or in a field environment, involving biological agents can also be accomplished by implementing decontamination procedures (20, 36, 67) that employ mechanical (physical) and chemical methods. The technical decontamination process for gross biological contamination is used to decontaminate vehicles and PPE. The medical or patient decontamination process is for cleaning injured or exposed individuals (67). The sequential nine-step technical decontamination process, used by the public service community, was developed in 1995 (51). Depending upon the contaminant, four cleaning solutions (known as A, B, C, and D) are used with water to effect decontamination. For technical biological decontamination, a 10% solution of sodium hypochlorite (Solution B) is used (67). A 10% solution of calcium hypochlorite is used as Solution B (52) because an effective concentration can be achieved in addition to a longer shelf life than that of sodium hypochlorite (M. S. Hildebrand, personal communication). Rosen (67) & Noll et al (52) describe details of this technical decontamination process. Decontamination of equipment or fabric clothing is accomplished with a 30-min contact with 5% sodium hypochlorite solution, followed by cleaning with soap and water. Because this procedure is corrosive to metal and fabrics, a thorough rinse with water is recommended after decontamination, followed by a process to preserve the treated item (20). Patient or medical decontamination is required when the biological agent (contaminant) places the exposed individual, or patient, at further risk or presents a potential secondary risk to other personnel. The emerging consensus (16, 40, 60) is that decontamination of persons exposed to a potential biological aerosol is probably unnecessary and that, at most, clothing removal and a soap and water shower are perfectly adequate to prevent secondary exposures. Based on the available evidence (36, 40), reaerosolization of biological agents from clothing or skin does not appear to be a major issue. Healthcare providers and first responders are not at risk from such a hazard in most circumstances.

The efficacy of decontamination of inanimate surfaces with liquid household bleach is documented in recent experiments conducted at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) (Hawley & Nash, unpublished observations). Undiluted liquid household bleach inactivated 99.8% of the spore population (a 5 $\log_{10}$ reduction in viability) of *B. anthracis* after 1 min of contact time. Similarly, after 1 min of contact, an *E. coli* population was completely inactivated (a 6 $\log_{10}$ reduction in viability). *E. coli* was used as a gram-negative model in these experiments to simulate *Y. pestis* and other gram-negative bacteria that are more fastidious in their growth requirements and are more dangerous to handle. Further experiments were conducted to determine the efficacy of 0.26% sodium hypochlorite (a 1:20 dilution of liquid household bleach, 2,625 parts per million free, available chlorine). Results showed a 100% inactivation of the spore population (a 5 $\log_{10}$ reduction in viability) of *B. anthracis* after 15 min of contact time to 0.26% sodium hypochlorite. Using 0.5% sodium hypochlorite (a 1:9.5 dilution of liquid household bleach, about 5,500 parts per million free, available chlorine), we observed a greater than 90% inactivation of the spore population (up to a 3 $\log_{10}$ reduction in viability) of *B. anthracis* after 5 minutes of contact time. Experiments are in progress to refine the contact time data for inactivating *B. anthracis* spores and to determine the influence of extraneous organic material on inactivation kinetics.

After an aerosol incident with a biological agent, the evolving belief is that there will be very minimal contamination of the clothing or skin of victims. In addition, decontaminating victims can be accomplished (if even necessary) by removing their clothing and having those potentially exposed shower at home with soap and water (16, 37, 40, 60). Reaerosolization of a biological agent in a hospital setting is unlikely (16, 23, 50, 65). The essential resources in providing support to first responders, law enforcement agencies, and the medical community to incidents involving biological weapons include microbiologists, biosafety professionals with a strong foundation in microbiology, and a designated clinical microbiology laboratory (72). The continuing education of medical responders is also an important component of this response network (14, 16, 56).

RESPONSE ISSUES

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In a field environment or during a response to a biological weapons incident, however, only personal protective measures, equipment, and decontamination procedures may be available to personnel. The Centers for Disease Control and Prevention (CDC) developed a strategic plan to address the deliberate dissemination of biological or chemical agents to reduce the vulnerability of the United States to biological and chemical terrorism. The plan includes preparedness planning, detection and surveillance, laboratory analysis, emergency response, and communication systems. For the diagnosis and characterization of biological and chemical agents, the CDC and its partners will create a multilevel laboratory response network for bioterrorism. The purpose is to link clinical laboratories to public health agencies in all states, districts, selected cities and countries, and to state-of-the-art facilities that can analyze biological agents. The CDC will transfer diagnostic technology to state health laboratories and others who will perform initial testing. They will also create an in-house rapid-response and advanced technology laboratory to provide around-the-clock diagnostic confirmatory and reference support for terrorism response teams (39). "Local" clinical microbiology laboratories will play an essential role in the initial recognition of a biological weapons incident. The laboratory capability will be challenged to apply procedures for the isolation, rapid detection, and subsequent identification (66) of a potential threat agent(s) in a suspect sample(s). Sample analysis data from the clinical microbiology laboratory will provide guidance to first responders and healthcare providers for environmental and expedient patient management, respectively. The medical community must be responsive to any biological weapons incident. Their response will include a prompt identification of the biological agent(s) by the clinical laboratory, or laboratory response network, and notification of local, state, and federal health and law enforcement agencies. They must also be able to provide support to healthcare providers who may be overwhelmed with caring for large numbers of infected or intoxicated casualties (42). In point of fact, medical examiners and coroners may be the first to recognize unusual deaths due to a biological weapons incident before the medical community becomes involved (53).

Both the microbiologist and biological safety professional should be able to apply most of the principles of biosafety and provide a practical assessment of the biological weapons incident

situation to responsible officials. Their knowledge is crucially important in helping to address microbiology and safety issues, minimizing fear and concerns of those responding to the incident, and helping to manage individuals potentially exposed to a threat agent. The biological safety professional will be able to apply the principles of biosafety in a practical approach to an incident involving a biological weapons agent.

ACKNOWLEDGMENTS

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Date: November 25, 2002

Enclosures: Copies of Brief (3)
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